

The Effects of Bifidobacterium Infantis 35624 on Inflammatory Markers and Exercise
Performance in Collegiate Female Swimmers

By

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Abstract

The purpose of this study was to determine the effects *Bifidobacterium infantis* 35624 (*B. infantis*35624) has on exercise performance, inflammation/immune function, and cognitive outlook during a six week exercise training phase in collegiate female swimmers. Using a two-group matched, double-blind, placebo-controlled design, seventeen NCAA Division 1 female swimmers were assigned to either group and supplemented daily for six weeks a 1×10^9 CFU dosage of *B. infantis*35624 (n=8) or placebo (n=9). Both groups underwent an intensive six week swim and resistance training program. Exercise testing (aerobic/anaerobic swim time trials and force plate vertical jump) as well as serum and salivary samples (cytokine and gastrointestinal (GI) inflammatory and immune markers) were collected pre, mid (week 3), and post (week 6). Recovery-stress questionnaire for athletes (RESTQ-Sport) was administered at baseline and at the end of each training week. Study data was analyzed by Analysis of Covariance (ANCOVA) by time point design with the respective baseline values of each dependent variable being the covariate. The *B. infantis*35624 group had a significant reduction in the anti-inflammatory marker IL-1ra ($p = 0.029$, $\eta_p^2 = .296$) and a noted statistical trend for a decrease in salivary IgA ($p = 0.060$, $\eta_p^2 = .231$) in comparison to placebo at mid-point. The *B. infantis*35624 group had significantly higher RESTQ-sport values in the sport recovery than the placebo group. *B. infantis*35624 in Division 1 female swimmers does not directly influence exercise performance, but did influence markers of inflammation/immune function as well as cognitive outlook during six weeks of exercise training.

Keywords: Probiotic, Swimmers, Exercise, Immune, Inflammation, Stress

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Chapter 1: Introduction

The relentless pursuit to improve exercise performance by athletes, coaches, exercise scientists, and sport dietitians has brought about extensive evaluation and understanding on the importance of proper nutrition. Fundamental aims for practicing sound nutrition are to promote good health, durability (i.e. decreased susceptibility to illness and/or injury) throughout intensified exercise training, enhance exercise training benefits (ex. power, speed, endurance, and recovery), and better optimize exercise performance. A nutritional practice often used with the intent to promote these desired benefits is dietary supplementation. One form of dietary supplementation known as probiotic supplementation has been attracting attention recently because of its effects on the gut microbiota and overall gut health [1].

The human gut microbiota contains a diverse bacterial species understood to exert numerous physiological functions such as protection against pathogens, barrier effects, regulation of energy levels and metabolism, neutralization of drugs and carcinogens, modulation of intestinal motility, and regulation of immunity within the entire gastrointestinal (GI) tract [2]. The gut microbiota is also believed to influence behavior and cognitive functions such as learning, memory, and decision-making [3]. Therefore, the impact on these various functions make it critical for the gut microbiota to exist in a state of “normobiosis”. Normobiosis is defined as a state where the beneficial bacterial microorganisms outweigh harmful species in order to maintain overall homeostasis and health [1]. Failure to preserve normobiosis can result in local and systemic inflammation due to gut bacteria microorganisms producing pro-inflammatory cytokines and stimulating an immune response [4].

Prolonged, intensive exercise training can potentially alter gut microbiota and disrupt “normobiosis” [5]. Exercise training can increase the risk of common GI complaints with

symptoms including nausea, stomach and intestinal cramps, vomiting, and diarrhea. These symptoms during exercise have been attributed to the physical up-and-down movement of the GI tract during running, shunting of blood flow from the GI to skeletal muscle, heart, or to the peripheral circulation for cooling purposes as well as thermal damage to the intestinal mucosa all causing intestinal barrier disruption [6, 7]. This increased permeability of the intestinal wall via damage to the mucosa will alter the gut microbiota, increase susceptibility to illness due to the absorption of antigens from microorganisms into tissue and blood stream and as a result stimulate an immune response, inflammation, and oxidative stress [8, 9].

Highly trained athletes such as elite swimmers undergo periods (> 1 week) of intensive exercise training (i.e. overreaching) which has been reported to result in an increased susceptibility of upper respiratory tract infections, GI symptoms, chronic exhaustion, and overall reductions in sport performance [9-12]. Overreaching is defined as short-term overtraining and is a state of fatigue induced by a period of repetitive, high volume intense microcycles of effort involving concentrated workloads with inadequate periods of physical rest [13]. It is hypothesized these overreaching intensive training phases undertaken by athletes such as swimmers increase the likelihood of microtrauma (i.e. injury) to muscle, connective tissue, and joints [14]. Consequently, these injuries are proposed to initiate a local and “whole body” systemic inflammatory response that can result in suppressed immune function and alter mood states [14]. These altered mood states in swimmers during increased training loads have been monitored and reported using the Recovery-Stress Questionnaire for Athletes (RESTQ-Sport) developed by Kellmann and Kallus [15]. The RESTQ-Sport model measures the athlete’s current perceived stress and recovery in a multidimensional approach to give insight into the stressing agents as well as the recovery strategies being implemented to better describe the

interrelations of stress-states and recovery demands. It has been reported that when a large exercise training load has been prescribed in swimmers (> 1 week) cognitive indicators of sport stress such as emotional exhaustion and injury were significantly increased while indicators of recovery such as physical recovery and being in shape were significantly reduced [16].

Probiotic supplementation has demonstrated to beneficially impact the intestinal mucosal barrier and improve GI integrity thus, in turn, strengthening GI immune response, reducing mucosal inflammation, and decreasing oxidative stress [17-22]. Recent findings have shown a specific probiotic strain termed *Bifidobacterium infantis* 35624 (*B. infantis* 35624) to positively impact immune response by inducing regulatory T cells (Treg) in both animal and human models within the gut in addition to beyond the gut [23-25]. Specifically beyond the gut, *B. infantis* 35624 supplementation has been shown to increase secretion of Treg cells in the peripheral blood of healthy human subjects [26]. Furthermore, 6-8 weeks of *B. infantis* 35624 supplementation has markedly decreased inflammatory biomarkers TNF- α and IL-6 in immune-inflammatory diseases such as chronic fatigue syndrome (CFS) patients as well as LPS stimulated peripheral blood mononuclear cells (PBMCs) in healthy subjects. These systemic reductions were suggested to occur due to an increased number of Treg cells [23]. This effect on systemic inflammatory response via regulatory T cell production with *B. infantis* 35624 supplementation presents an interesting finding for athletes, such as swimmers, who participate in chronic, prolonged intensified exercise training resulting in potential changes in systemic inflammation, compromised immune function, high levels of fatigue, and periods of cognitive stress [27]. Presently, it is not known if *B. infantis* 35624 supplementation in swimmers can influence inflammation, exercise performance, and mood states during rigorous intensive training phases [16].

Purpose

The purpose of this research study is to examine if chronic *B. infantis* 35624 supplementation throughout an intensified six week exercise training phase influences systemic inflammation, exercise performance, and cognitive outlook.

Rationale and experimental approach to the problem

If intensified exercise training can potentially disrupt “normobiosis” in the gut microbiome resulting in local (i.e. GI) and systemic inflammation then it is important to find an intervention capable of better controlling said inflammation from such exercise training. Due to the recent findings previously discussed regarding *B. infantis* 35624 probiotic supplementation on immune and GI/systemic inflammation as well as probiotics on cognitive function it appears this specific strain could be an appropriate intervention for healthy athletes undergoing intensified exercise training. If *B. infantis* 35624 can positively influence exercise induced inflammation then it will be important to understand if there is a relationship between changes in gut derived systemic inflammation, whole body exercise performance, and cognitive outlook during the training phase.

To determine the effects of *B. infantis* 35624 on the inflammatory response, exercise performance, and cognitive outlook during an intensified exercise phase, a double-blind, randomized placebo controlled Analysis of Covariance (ANCOVA) by time point design with the respective baseline values of each dependent variable be the covariate was utilized. Table 1 illustrates the general research design and procedures. The dependent variables for this study included markers of systemic inflammation, immune, and gut integrity as well as measures of exercise performance (500m aerobic swim test, 100m anaerobic swim test, vertical jump force

plate test) and cognitive response using the Recovery-Stress Questionnaire for Athletes (RESTQ-52 Sport). Participants who met the inclusion criteria were randomly assigned to ingest either B. infantis 35624 or placebo. Supplementation commenced at the beginning of the planned intensified training phase thus requiring baseline measurements which included blood samples and exercise performance testing to be completed at least 72 hours prior to start. Additional blood samples and exercise testing to determine possible inflammation and performance variations during respective training phase occurred at midpoint and immediately upon completion of said phase.

Independent Variables

Subjects recruited to participate in this study were Division 1 collegiate female swimmers. All subjects had a relatively similar training history at start of study since it was at the conclusion of their competitive season followed by two weeks of physical recovery. They were instructed to ingest a daily oral encapsulated supplementation regime of either 4mg (1 Billion count) B.infantis 35624 or 4mg of maltodextrin as the placebo throughout entire study.

Dependent Variables

Dependent variables included exercise performance testing, blood and salivary sampling to measure inflammatory and immune markers, and a cognitive stress-recovery assessment. Exercise performance testing occurred at three separate times (pre, mid, and post) during the designated supplementation and training phase. Testing included a vertical jump force plate test as well as an aerobic and anaerobic swim-specific performance assessment. The force plate was utilized in accordance with a standing vertical jump movement to measure rate of force development and explosive power. The vertical jump test using a force plate was already being

conducted by the team on a monthly basis during the competitive season therefore familiarization was not be required. The aerobic swim test was a 500 meter freestyle time trial and a anaerobic swim test consisting of a 100m prime freestyle time trial. Each swim test was conducted in a 25 meter training pool located in team's home natatorium. Both swim tests are standard assessments of exercise performance frequently conducted by the team's head coach during the competitive season thus the exercise performance testing did not require familiarization prior to experiment.

In accordance with the exercise performance testing, blood and salivary samples were also obtained pre, mid, and post of the supplementation/intensified training experiment. These samples provided gut inflammatory markers (LPS and LPS Binding Protein) as well as immunoglobulin A (IgA) and cytokine immune markers (IFN- γ , IL-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-17F, IL-22, TNF- α). Outside the pre-supplementation baseline sample prior to the onset of intensified training, both mid and post blood/salivary samples were taken in a rested state outside of exercise training during training weeks 3 and week 6 respectively. Serum samples were allowed to clot at room temperature. The serum was then separated by refrigerated centrifugation and subsequently aliquoted for immediate assay or frozen at -80°C for later analyses according to assay procedure requirements. Assays included a 13-plex cytokine panel, LPS/LBP, and salivary IgA. These assays were performed at all blood collection time points previously mentioned.

A cognitive stress-recovery assessment using the Recovery-Stress Questionnaire for Athletes (RESTQ-Sport) was utilized to determine the participant's cognitive outlook throughout the intensified training study [15]. The primary aim for using the RESTQ-Sport was to help measure each supplementation group's level of stress and recovery during the intensive training

experiment. More specifically, it is additional speculative work to see if the probiotic supplementation could potentially influence the extent to which a participant is physically and/or mentally stressed which, in theory, could provide additional evidence as to why exercise performances might be different between the two groups. The RESTQ-Sport was completed by all participants at baseline as well as at the conclusion of each training week (weeks 1-6).

Hypotheses

Inflammation. Hypothesis 1: *B. infantis* 35624 supplementation will effect blood markers of inflammation within the gut and beyond the gut as well as enhance immune function during an intensified exercise training phase compared to placebo.

Exercise Performance. Hypothesis 2: *B. infantis* 35624 supplementation will enhance both aerobic and anaerobic swimming exercise performance measures as well as vertical jump force plate testing during an intensified exercise training phase compared to placebo.

Psychological Assessment. Hypothesis 3: *B. infantis* 35624 supplementation will positively impact and improve measures of cognitive stress and recovery during an intensified training phase compared to placebo.

Limitations and Delimitations

One key limitation to this research study will be the total number of subjects recruited. Since our subject pool will be derived from a Division 1 collegiate female swim team our total expected number of subjects will be approximately 20. There are 22 female swimmers on the

team and we expect to lose, hopefully, only a couple due to not meeting subject inclusion criteria or just declining not to be a participant in the study (i.e. exiting seniors). This sample size could impact our statistical power and reduce the likelihood that we will reject the null hypothesis and detect a statistically significant effect of *B. infantis* 35624 supplementation on inflammation markers, exercise performance, and cognitive response measures.

An important delimitation will be that we are not measuring exercise volume and intensity throughout the six week “intensified exercise training phase” to ensure an appropriate physical stimulus. Rather, we are simply trusting the team’s head coach and strength & conditioning coach that the implemented training phase in the pool and weight room will induce higher levels of physical fatigue resulting in decrements of exercise performance during the study.

A second delimitation is not including additional athlete subject populations. This is due to the intensified exercise training phase in both the natatorium and weight room being extremely specialized for Division 1 collegiate female swimmers which will require extensive familiarization prior to beginning of the study and not practically feasible to achieve in other athlete/sport populations.

A final delimitation made for the study is not measuring fecal excretion of *B. infantis* 35624 to verify the desired changes in fecal microbiota. Previous research using *B. infantis* 35624 supplementation has been able to confirm detection of its bacterial DNA in fecal samples suggesting the probiotic is able to survive transit through the GI tract [28]. Therefore, to help keep study’s attention strictly on *B. infantis* 35624 effects on inflammation, exercise performance, and cognitive response we will refer to these previous findings in regards to the probiotic’s ability to survive the entire GI tract.

Assumptions

We assume the team's head coach and strength and conditioning coach are providing an appropriate exercise stimulus that is overreaching which should be sufficiently intensive and exhaustive enough to subsequently decrease exercise performance during the research study. In addition, we assume the head coach and strength coach will set clear expectations on how the subjects are to complete each exercise training session and not permit subjects to give poor effort.

We assume the subjects will be truthful with inclusion criteria at the start of study and appropriately notify us if any aspects of inclusion changes anytime during the study to help control potential external influences on findings. We also assume subjects are giving their best effort during the entire exercise study, remain truthful each week when completing the RESTQ-Sport, and maintain a relatively normal dietary intake.

Finally, we are assuming the *B. infantis* 35624 supplement we purchase at a local retail store does indeed contain the 1 billion count of viable bacteria (4mg).

Table 1 General research procedures

		Week 0 Baseline	Weeks 1&2 8hr	Week 3 8hr	Week 4 8hr	Week 5 20hr	Week 6 20hr
Monday	AM PM		Week 1 Compliance	Week 2 Compliance	Week 3 Compliance	Week 4 Compliance	Week 5 Compliance
Tuesday	AM PM	100m Swim		100m Swim			100m Swim
Wednesday	AM PM	Force Plate Blood sample		Force Plate Blood sample			Force Plate Blood sample
Thursday	AM PM	500m Swim		500m Swim			500m Swim
Friday	AM PM	Return Logs RESTQ52	RESTQ52	Return Logs RESTQ52	RESTQ52	RESTQ52	Return Logs RESTQ52

Chapter 2: Literature Review

B. Infantis 35624

Discovery. *B. infantis* 35624 was first discovered from resected healthy human gastrointestinal tissue approximately 20 years ago [24]. The discovery was result of a bigger project aimed to take healthy human gastrointestinal (GI) tissue and isolate/identify probiotic candidates that were populated with lactic acid bacteria. It was hypothesized this co-occupancy of probiotic strains, like *B. infantis* 35624, with lactic acid bacteria amongst the mucosa of resected GI tissue would identify probiotic strains that could not only work well in the complex human GI system, but also have potential GI immunomodulatory effects on epithelial cells, dendritic cells, and T lymphocytes.

B. Infantis 35624 Immunomodulatory Effects

GI Transit. Before *B. infantis* 35624 can begin to exert its potential GI immunomodulatory effects it must first survive degradation throughout the human GI tract, particularly by the gastric acid and bile salts in the upper GI tract. A fecal excretion study is commonly applied to understand a probiotics ability to survive GI transit. The measurement of a fecal sample's microbiota during probiotic supplementation using quantitative PCR (qPCR) will provide recovery rates of the supplemented probiotic's DNA as well as help gain insight on the potential influence probiotic supplementation is having on the composition of the GI microbiota.

A fecal excretion study in humans has been conducted with *B. infantis* 35624 supplementation by Charbonneau et al. 2013 [28]. This study was conducted by supplementing two groups, an irritable bowel syndrome (IBS) diseased group and a healthy group, with a 1 billion count of oral supplemented *B. infantis* 35624 daily for eight weeks. They found in both

groups from Week 1 to Week 4 of supplementation similar increases in fecal excretion of *B. infantis* 35624 DNA with steady state being reached between Week 4 to Week 8. Thus, it appears in both a healthy and non-healthy GI, *B. infantis* 35624 can survive GI transit. However, a limitation of this study is the PCR only detected *B. infantis* 35624 DNA and did not perform strain selective culture techniques to confirm the microbe's viability in the feces.

Epithelial Cells. Epithelial cells are located within the GI mucosa and are the first to encounter microbes. The epithelium is critical in maintaining intestinal homeostasis and does so by actively sampling pathogens, antigens, and resident bacteria [29, 30]. Certain microbes' epithelial cells encounter will induce pro-inflammatory transcription factor expression and chemokine secretion. For example, in response to pathogenic bacteria, pro-inflammatory cytokines such as tumour necrosis factor (TNF- α) as well as a pro-inflammatory chemokine, interleukin 8 (IL-8), are secreted by epithelial cells and transcriptionally regulated by nuclear factor (NF)- κ B, a pro-inflammatory signaling pathway [31-33].

Epithelial cells exposed to the commensal bacteria *B. infantis* 35624 have been demonstrated by O'Hara et al. 2006 to be immunologically unresponsive [34]. O'Hara et al. found *B. infantis* 35624 does not induce pro-inflammatory responses by the epithelial cells and instead inhibits epithelial cell secretion of the pro-inflammatory chemokine IL-8. Moreover, they reported that NF κ B activation via *Salmonella typhimurium* and subsequent IL-8 secretion was attenuated by *B. infantis* 35624. Similar findings were observed in 2009 by Sibartie et al. using human intestinal epithelial cells. Sibartie et al. reported that when exposed to several pathogens (i.e. *Salmonella typhimurium*, *Clostridium difficile*, *Mycobacterium paratuberculosis*) the epithelial cells activated NF κ B and stimulated secretion of the pro-inflammatory chemokines

CCL20 and IL-8 [35]. However, when the intestinal epithelial cells were exposed to *B. infantis* 35624 they did not secrete CCL20 thus limiting inflammatory signals induced by pathogenic bacteria. Therefore, both O'Hara et al. and Sibartie et al. have demonstrated epithelial cells to be immunologically dormant when exposed to *B. infantis* 35624. Furthermore, it appears *B. infantis* 35624 could elicit immunomodulatory effects on the epithelial cell's pro-inflammatory response to gastrointestinal pathogens.

Dendritic Cells. Dendritic cells (DCs) are cells present in areas highly vulnerable to pathogenic entry such as the GI system. They are professional antigen cells whom survey their environment looking for antigenic material. Once antigenic material is located the DCs convert from immature sentinel cells into mature, potent effector DCs. These mature DCs will then migrate to T-cell areas to activate and influence T-cell pathogen-specific cytokine expression levels [36].

According to O'Mahony et al. 2006, DCs has a divergent cytokine response to *B. infantis* 35624 in comparison to pathogenic bacteria [37]. They utilized DCs isolated from human mesenteric lymph node following a surgical resection of inflamed gut as a result of inflammatory bowel disease. O'Mahony et al. found these DCs, in response to *B. infantis* 35624, stimulated secretions of IL-10 and TGF β indicating a reduction in inflammation. This finding was in stark contrast to the same DCs exposed to the pathogen bacteria *Salmonella typhimurium* which induced only pro-inflammatory markers IL-12 and TNF α . Similar findings were reported by O'Hara et al. 2006 in which *B. infantis* 35624 stimulated the secretion of IL-10 and TNF- α by myeloid DCs [34]. Additional findings by Konieczna et al. 2012 helped confirm these divergent DCs cytokine specific secretions by stimulating numerous other DCs subsets with *B. infantis* 35624 resulting in secretion of only IL-10 and not IL-12 [26].

It was reported the same year by Gad et al. 2011 that the effects of *B. infantis* 35624 on human dendritic cells were further reaching [38]. Gad et al. demonstrated the ability of *B. infantis* 35624 to actually suppress DCs derived IL-12 secretion while exposing these DCs to three different pro-inflammatory cocktails aimed to induce IL-12. These suppression levels were stated to be comparable with 1.0 μ M dexamethasone, an anti-inflammatory glucocorticoid known to inhibit IL-12 secretion. They also found similar *B. infantis* 35624 effects on DC IL-10 secretion, but stated the IL-10 secretion was not involved in the IL-12 suppression. This was determined by adding a neutralizing anti-body toward IL-10 and still observed IL-12 suppressive activity in the presence of *B. infantis* 35624. Thus, the current literature suggests *B. infantis* 35624 can both selectively stimulate an anti-inflammatory cytokine response and suppress a pro-inflammatory cytokine response in numerous human DCs.

T Lymphocytes. One kind of T lymphocyte known as regulatory T cells (Treg) is part of the immune response designated to better contain reactions and limit the aggressive spread of these immune responses to other tissues. Treg cells can be induced from the gut mucosa [39]. They are integral in preserving tolerance to gastrointestinal microbiota and food antigens as well as influencing immunological reactivity to said microbiota and antigens [39-41]. These Treg cells within the gut mucosa are induced primarily from DCs which suggest commensal bacteria could influence Treg production per previous evidence of *B. infantis* 35624 on DCs [42].

B. infantis 35624 in the animal model has been shown to induce Treg cells and mediate protection against a pathogen stimulated pro-inflammatory response [43]. O'Mahony et al. 2008 exposed mice to either the pathogen *salmonella* or lipopolysaccharide (LPS) which is an endotoxin known to illicit a strong immune response in animals. They found the mice fed with

B. infantis 35624 throughout the course of either infection significantly increased Treg cell production. This *B. infantis* 35624 derived inductions of Treg cells in the small intestine, liver, and spleen of infected mice reduced the expression of the pro-inflammatory transcription factor NF κ B. The impact of a reduced NF κ B response resulted in a reduction of pro-inflammatory cytokines such as TNF- α and IL-6 in the small intestine, liver, and spleen. Therefore, it appears *B. infantis* 35624 induced Treg cells can influence pro-inflammatory pathways in response to a translocating pathogen.

Additional findings by Konieczna et al. 2012 reported human DCs stimulated with *B. infantis* 35624 were shown to selectively upregulate forkhead box P3 (Foxp3) expression in immature lymphocytes and subsequently induce Treg cells which are vital in protecting against LPS or pathogen-induced NF κ B activation [26]. Foxp3 is known as a master transcription factor critical in the development/function of Treg cells and in maintaining overall immune homeostasis [44]. This increased expression of Foxp3 Treg cells are found in peripheral blood and is associated with an increased secretion of IL-10. These peripheral Treg cell findings by Konieczna et al. were later supported by Groeger et al. 2013 in both healthy humans and humans diagnosed with chronic fatigue syndrome (CFS) [23]. Groeger et al. found that CFS patients supplemented with a 10 Billion count of *B. infantis* 35624 supplementation daily for 6-8 weeks exerted significant reductions in TNF- α and marginal decreases in IL-6 levels systemically. Furthermore, ex vivo studies from LPS stimulated peripheral blood mononuclear cells in the healthy human subjects also resulted in significant reductions in IL-6 and TNF- α secretion. These findings suggest *B. infantis* 35624 supplementation in both the animal and human model can induce DC derived Treg cell production to provide additional immunomodulatory effects both within the gut and systemically beyond the gut.

Neural Function – Gut-Brain Interaction. The strong evidence demonstrating *B. infantis* 35624 immunomodulatory effects within the gut and systemically beyond the gut has given way to research efforts aimed in understanding these effects on gut-brain interactions. For example, in the animal model, it has been reported that in the absence of GI microbes there is a reduced expression of brain-derived neurotrophic factor within the cortex and hippocampus with an exaggerated hypothalamic-pituitary-adrenal axis in response to stress [45]. However, chronic supplementation of probiotic bacteria in rats has shown to attenuate the cytokine response under mitogen stimulation which is known to activate different signaling pathways in early and late-divided T cells [46]. In addition to these cytokine reductions, probiotic supplementation has also been able to induce increases in peripheral concentrations of the serotonin precursor tryptophan [46]. Interestingly, a clinical trial conducted by Rao et al. 2009 has demonstrated an increase in the concentration of *Bifidobacterium* within the gut can reduce levels of anxiety and improve mood in CFS patients [47]. Therefore, these findings suggest probiotic bacteria can positively influence various emotional states.

Bifidobacteria has been reported in the animal model to be vulnerable to the effects of emotional stress and negatively alter the composition of intestinal microbiota resulting in reduced concentrations of *bifidobacteria* [48, 49]. To study the specific effects of *B. infantis* 35624 on stress, Desbonnet et al. 2010 utilized the maternal separation (MS) model of depression in rodents [50]. Desbonnet et al. found that rodents chronically supplemented with *B. infantis* 35624 had attenuated MS symptoms. Typical MS symptoms included in this study decreased forced swim test performance followed by an increase in immobility during the forced swim test as well as exaggerated MS derived IL-6 release. Although, these attenuations were not of the

same magnitude as the MS rodents treated with the anti-depressant citalopram. Nonetheless, this study provides preliminary evidence suggesting chronic *B. infantis* 35624 supplementation in the MS rodent depression model can positively influence neuronal systems and behaviors pertinent depression. These findings in addition to Rao et al. in CFS patients presents a potentially bigger role *Bifidobacterium* and its subspecies *B. infantis* 35624 can play in gut-brain interactions as well as suggests a prospective therapeutic application for these probiotics in response to various forms of stress.

Acute Intensive Exercise (Stress)

Pro-inflammatory Cytokine Response - TNF- α and IL-1 β . Circulating levels of pro-inflammatory cytokines TNF- α and IL-1 β have been reported to increase during and after exercise [51-55]. These responses have been measured in various exercise modes. For example, circulating TNF- α levels significantly increased post-exercise (immediately or 2-3hr post-exercise) in near-maximal intensity exercise under 30 minutes in duration, submaximal concentric exercise within 2hr in duration (ex. cycling, rowing, running), and endurance exercise lasting longer than 2hrs [51, 53, 56, 57]. However, numerous other studies implementing analogous exercise modes were unable to report similar increases in circulating TNF- α after exercise [58]. This inability to detect TNF- α consistently has been suggested to be attributed to either the exercise intensity being too low, a delayed secretion, or a rapid clearance from circulation into the urine since the kidney is the major organ for TNF- α clearance [59, 60]. Furthermore, soluble TNF receptors have been shown to be stimulated by exercise which can also inactivate potential action of TNF- α [59]. Therefore, according to the literature, circulating TNF- α levels following acute exercise training can be affected by numerous mechanisms resulting in inconsistent systemic responses.

Similar to TNF- α , circulating levels of IL-1 β have been demonstrated to increase in response to near-maximal intensity exercise under 30 minutes duration or endurance exercise longer than 2 hours in duration [54, 55, 61, 62]. However, the large majority of studies using similar exercise protocols have failed to report comparable findings [58]. Significantly increased circulating levels of IL-1 β were predominately found to occur after long-duration exercise [55, 61, 62]. It is believed this inability to consistently detect circulating IL-1 β is due to a delayed secretion, short half-life (6-10 minute), lack of highly sensitive methodologies necessary to detect its systemic concentrations changes which occur in the pg/ml range, or its increased circulation remains localized in injured tissue and does not leak out systemically unless damage is too great [63]. Consequently, despite limited findings reporting increased circulating levels of the pro-inflammatory cytokines TNF- α and IL-1 β in response to an acute exercise bout, it appears that multiple mechanisms during/immediately post-exercise as well as varying exercise intensities/durations are strongly influencing changes on a systemic level resulting in changes to occur mainly in local tissue, hence, a conflict in findings amongst the literature.

Immunomodulatory Cytokine Response – IFN- γ . Interferon gamma (IFN- γ) is an immunomodulatory cytokine shown in numerous studies to decrease in response to exercise [64-68]. Reduced production of IFN- γ following exercise was observed to occur in peripheral blood monocytes, lymphocytes (ex. Treg cells) or whole blood. A study by Clancy et al. 2006 found the secretion of IFN- γ from blood CD4 T cells measured in whole blood culture was significantly lower in athletes reporting fatigue in comparison to healthy controls [65]. Clancy et al.'s finding supports previous studies demonstrating exercise (acute and chronic) to downregulate IFN- γ secretion by immune cells such as lymphocytes. It also suggests these

altered productions by these particular immune cells can negatively modulate immune function potentially increasing susceptibility to fatigue and infection.

Multi-functional Cytokine Response – IL-6. The multi-functional cytokine IL-6 has been reported to increase following various modes of exercise such as near-maximal exercise, submaximal concentric exercise less than two hours in duration, eccentric exercise, and endurance exercise greater than two hours in duration [51, 52, 55, 60, 69-75]. IL-6 responses to near-maximal exercise and submaximal concentric exercise ranged from 25% - 75% increases on up to 2-5 times higher circulating levels in comparison to pre values while eccentric exercise produced slightly greater responses ranging between 5-7.7 times higher than pre values [51, 60, 70, 72, 73, 75]. IL-6 responses to endurance exercise, such as marathon and ultramarathon running, was significantly higher with ranges between 30 – 128 times higher than pre values [27, 55, 76-78]. Circulating sources of IL-6 are typically from blood leukocytes (i.e. monocytes), the brain, and liver. However, during intensive bouts of exercise, skeletal muscle has been shown to significantly contribute to these substantial increases [79]. Significant increases of IL-6 in response to exercise are transient with levels returning to baseline resting levels within an hour post-exercise [80]. These findings suggest IL-6's response to exercise is driven more by duration rather intensity with the longer duration exercise bouts producing the higher systemic IL-6 circulating levels resulting in an emergency recruitment of neutrophils and subsequent inflammatory response [58].

Anti-inflammatory Cytokine Response - IL-1Ra. IL-1Ra is an anti-inflammatory cytokine and is a natural antagonist that competes with IL-1 β for receptor binding without initiating signal transduction [81, 82]. Significant elevations in IL-1Ra levels of up to 40 fold increases have been reported to occur following most endurance exercise [58]. However, following higher intensity endurance exercise, such as a marathon race, IL-1Ra levels have been reported to be 200 times greater than resting levels [83]. Research suggests this increase following endurance exercise is in response to the rapid increase of IL-6 concentrations [27, 84]. Previous studies have demonstrated IL-1Ra originates from mononuclear and polymorphonuclear leukocytes as opposed to IL-6 which is produced locally in the working muscle during exercise [77, 84, 85]. It is believed the production of the pro-inflammatory cytokine IL-6 within skeletal muscle is increased during/after exercise and capable of being released in large amounts into the circulation. As a result of this systemic release of IL-6 from skeletal muscle, leukocyte derived IL-1Ra production is significantly increased in efforts to block the IL-1 receptor and exert strong anti-inflammatory effects [84, 86, 87]. Thus, it is suggested the levels of systemic IL-1Ra reflect the production of IL-6 [88].

Anti-inflammatory Cytokine Response – IL-10. According to the literature, the anti-inflammatory cytokine IL-10 has been reported to significantly increase following only exhaustive endurance exercise greater than two hours in duration [27, 55, 67, 76, 89]. These exhaustive endurance exercise bouts included completions of marathon and ultramarathon races resulting in IL-10 levels to be 27 – 60 times higher in comparison to pre-exercise levels. It is believed these increased circulatory levels of IL-10 are in response to the transient rise in circulating IL-6 [90]. This association between IL-6 and IL-10 was demonstrated in a study by

Steensberg et al. 2003 that intravenous infused IL-6 could mimic the acute rise in IL-10 as observed following a bout of exercise in humans. These increased levels of IL-10 are believed to be the cause of immunosuppression associated with this kind of physical trauma by attenuating the synthesis of cytokines such as IL-1 β , TNF- α , and IL-6, resulting in an increased susceptibility to infection or illness [91, 92].

LPS Response. Increased concentrations of LPS in the blood have been associated with an acute intense bout of endurance exercise [52, 93]. LPS is a gram-negative bacterial endotoxin normally located within the intestine. In normal (non-exercise) conditions, the tight junctions at the epithelium of the intestine are essentially impermeable to gram negative bacteria such as LPS. However, undergoing intense exercise will redistribute blood supply thus reducing blood flow to intestinal mucosa resulting in hypoperfusion and as a result compromise the intestinal mucosa wall [94]. Consequently, this damage to the intestinal mucosa wall allows LPS to translocate into the bloodstream during intensive exercise [93]. Increased concentrations of LPS in the bloodstream activate inflammation by increasing production of pro-inflammatory cytokines such as TNF- α and IL-6.

Exercise-induced changes in plasma LPS concentrations have been shown to be affected by ambient temperature (i.e. hot vs cold). A study by Yeh et al. 2013 found in healthy subjects completing a 45 minute treadmill exercise at 70% $\text{VO}_{2\text{max}}$ in a climate chamber at either 91° F and 50% humidity or 77° F at 60% humidity plasma LPS concentrations were significantly higher in the heat with no change in the cool exercise trial [95]. However, they reported intestinal permeability responded similar in both hot and cool environments. Yeh et al. suggest the higher LPS plasma concentrations in a hot environment are not because of a difference in

intestinal permeability, but instead due to a compromise in the ability to remove LPS by anti-LPS mechanisms in the blood.

These observed changes in plasma LPS concentrations following intensive exercise produce cytokines in response to this endotoxin. A study by Abbasi et al. 2013 examined the effects prolonged exhaustive exercise on LPS-induced cytokine production in whole blood [96]. Abbasi et al. used well trained men and women and had them perform an official half-marathon in a cool temperature environment of 33° F. They collected whole blood samples before, 30 minutes after, 3 h after, and 24h after the half marathon. The cytokines included in this study were TNF- α , IFN- γ , and IL-10. In regards to TNF- α , Abbasi reported no induction by the exercise bout, but the subject's LPS-stimulated blood samples production of TNF- α was significantly lower in comparison pre-exercise levels with no difference between sexes. Another pro-inflammatory cytokine, IFN- γ , also had a substantial suppression following LPS stimulation. Therefore, it appears these pro-inflammatory cytokine productions were significantly downregulated following intensive exercise. Of particular interest was their findings with IL-10. They reported IL-10 to be most strongly influenced by the exhaustive exercise. IL-10 in unstimulated control cultures had a significant increase 30 minutes following the run. However, it was noted these exercise-induced IL-10 levels 30 minutes post-exercise were only significantly higher in men and not women. In addition, under LPS-stimulation, they did not observe a significant increase in IL-10 production in either sex. This finding suggests exercise induction of IL-10 operates through entirely different pathways and not by LPS-stimulation. Furthermore, these results potentially indicate a less pronounced anti-inflammatory cytokine response in women as compared to men following intensive prolonged exercise.

Treg Cells Response. Foxp3 Treg cells has been shown in response to *in vitro* antigenic stimulation following a 12 week Tai Chi Chaun exercise program to markedly increase production of TGF- β and IL-10 in human peripheral blood mononuclear cells (PBMCs) [97]. In a mouse model study using a moderate- or high-intensity running exercise program reported that high-intensity training increased Treg cell numbers with subsequent reductions in pro-inflammatory (IL-2, IFN- γ , and IL-12) and increased anti-inflammatory cytokine expression (IL-1ra, IL-4, and IL-10) [98]. These findings suggest high-intensity exercise as well as chronic exercise training can mobilize and boost Treg cell production derived from lymphoid organs such as the spleen thus increasing anti-inflammatory cytokine secretions such as IL-10.

Chronic Intensive Exercise

Immune Cell Response. Monocytes, neutrophils, and DC are functionally important immune cells understood to produce various inflammatory cytokines such as IL-1 β , IL-6, and TNF- α in efforts to protect against infections. These immune cell derived cytokines have been shown by Morgado et al. 2012 to be affected by chronic, prolonged intensified exercise training phases in a group of elite swimmers [99]. Morgado et al. obtained peripheral blood samples on four separate occasions throughout their intensive training season and found that chronic, long term intensive training significantly effects the capacity of the immune cells (i.e. monocytes, neutrophils, and DC) to produce inflammatory cytokines. This was determined by measuring immune cell derived cytokine production following 6 hour incubation in the presence or absence of inflammatory stimulates LPS and IFN- γ . That is, LPS and IFN- γ stimulation decreased immune cell cytokine production during the training season. A reduction in these immune cell cytokines could increase susceptibility to infection and illness.

Immunoglobulin A (IgA). IgA is a class of antibodies secreted in fluids such as saliva, tears or mucus from the intestines. It is the predominant protein in the mucosal antibody response which is critical in helping neutralize toxins and remove pathogens [100]. However, chronic, prolonged intensive exercise has been shown to decrease salivary IgA levels and subsequently increase likelihood of developing an upper respiratory tract infection in athletes [101, 102]. This inverse relationship between salivary IgA and development of upper respiratory tract infection was demonstrated in marathon runners by Nieman et al. 2006 [103]. They found salivary IgA secretion was reduced by 10% with 25% of the runners reporting an upper respiratory tract infection within two weeks after completing the race. It has been suggested by Steerenberg et al. 1997 this increased susceptibility to an infection after prolonged intensive exercise could be due to the exposure of micro-organisms or pathogens present in the air or water in concert with a weakened antibody immune response [104].

Cognitive Response. Chronic intensive exercise training is utilized by competitive athletes to help enhance sport and/or exercise performance. It is common for well-trained athletes to undergo short intensive exercise training microcycles known as “overreaching” phases to induce a state of fatigue as a result of concentrated exercise workloads with minimal time for adequate rest and recovery. This overreaching phase is often associated with decreased exercise performance. In addition to whole-body fatigue and diminished performance, cognitive response during an intensive overreaching phase has been reported to be negatively affected [16, 105]. Changes in mental state (i.e. enhancement of negative moods), emotional instability, drop in motivation, and even depression have all been associated with overreaching or overtraining loads [106-110]. These alterations in cognitive outlook during chronic intensive exercise training

cycles are believed to be strongly influenced by non-specific immune activation and systemic cytokine release [14, 105, 111].

To better understand the relationship between chronic intensive exercise training and cytokines, a study by Main et al. 2010 utilized elite male rowers preparing for the 2007 Rowing World Championships [105]. These athletes trained seven days a week which included 14 training sessions each week resulting in a weekly training duration of 24 hours (3.4 hours/day). Throughout the eight week study they collected blood samples to analyze cytokine values on 4 separate occasions in addition to a 22-item training distress assessment model. Main et al. found that during the eight weeks of intensive endurance training leading up to the championships symptoms of overreaching were observed, but none of the athletes developed overtraining. Despite no statistically significant changes during the overreaching phase for any of the cytokines measured (IL-1 β , IL-6, IL-8, IL-10, TNF- α , IL-12p70), changes in inflammatory cytokines on measures of training overload (i.e. training distress) discovered significant effects. For example, there was an association between increased measures of depressed moods and significant increases in IL-1 β , IL-6, and TNF- α . Furthermore, increases in depressed moods were also associated with a significant decrease in IL-10. Perceived overall stress was associated with a significant increase in IL-1 β and TNF- α . Finally, increase in vigor was associated with decreases in IL-6 and an increase in IL-10. Therefore, these findings by Main et al. suggest a potential relationship between changes in systemic cytokines and cognitive response during chronic intensive exercise training.

One model utilized to measure cognitive response during phases of chronic intensive exercise training is the Recovery-Stress Questionnaire for Athletes (RESTQ-Sport) [15]. This questionnaire consists of either 76 (long version) or 52 (short version) items used to assess in a

multidimensional way an athlete's recovery-stress state during a specific training cycle. Each item answered in the RESTQ-Sport will indicate how often an athlete participated in certain activities during the previous three days and nights. These items are used to generate 19 specific recovery-stress scales which are grouped into four categories; general stress, general recovery, sport stress, and sport recovery. The test-retest reliability of each scale in numerous sport research samples (ex. swimming, rowing, and track & field) using Cronbach alpha coefficients has demonstrated acceptable internal consistency with values slightly less or greater than 0.7 if repeated every 3-4 days [15, 112]. In addition, the expected relationships between each RESTQ-Sport scale and the actual state have been empirically verified by its correlation with other models such as the Multidimensional Physical Symptom List, the Profile of Mood States, and the State-Trait-Anxiety Inventory [15].

The RESTQ-Sport derived recovery-stress state has also demonstrated an ability to reflect training load. For example, multiple RESTQ-Sport studies using samples from sports such as rowing, swimming, soccer, basketball, and mountain biking have reported a dose-response relationship between the respective sport's training volume and the subjective assessment of stress and recovery [15, 16]. Therefore, these findings suggest RESTQ-Sport provides the reliability and validity necessary to effectively observe the cognitive response of individual athletes and/or sport teams over periods of chronic intensive exercise training.

Probiotic Supplementation and Athletes

Immune Function. According to a commentary article by Pyne et al. 2015, since 2006, 15 experimental studies have been published investigating the effects of probiotics on athletes [113]. The most commonly studied probiotic species used in these probiotic and athlete studies was *Lactobacillus* [113]. The primary aims of these studies were to determine the influence

probiotics might have on clinical measures of illness and immune function. For example, a study by Clancy et al. 2006 found in fatigued athletes with impaired athletic performances their whole-blood culture level of IFN- γ from Treg cells was approximately half of the healthy control athletes thus suggesting an immune defect [65]. However, after Clancy et al. supplemented the fatigued athletes with a 10 Billion count of *L. acidophilus* daily for four weeks they found the fatigued athletes Treg cell derived IFN- γ secretion was restored. This was the first study to report in fatigued athletes a systemic Treg cell immune defect could be reversed with probiotic supplementation and potentially decrease illness susceptibility. This whole-blood IFN- γ increase with probiotic supplementation was supported by Cox et al. 2010 who reported that one month supplementation of *L. fermentum* (12 Billion count daily) in 20 elite male distance runners increased whole-blood IFN- γ secretion twofold with a 50% lower number of self-reported days of being ill (i.e. symptoms of upper respiratory illness) despite no statistically significant difference in salivary IgA [114].

GI Effects. As previously discussed, intense strenuous exercise can disrupt the intestinal mucosa wall leading to a more relaxed tight junctions and increased intestinal permeability. Probiotic supplementation has been proposed to potentially counteract exercise-induced intestinal barrier dysfunction [5]. This effect was hypothesized to occur by Lamprecht et al. 2013 which believed the supplemented probiotic population would surpass bacteria that activated zonulin production. Zonulin is a protein understood to modulate the permeability of the tight junctions. Increased levels of zonulin means permeability is greater. Lamprecht et al. found ingestion of a multi-species probiotic supplementation (10 Billion count/day) for 14 weeks in trained men following a triple-step cycle ergometry exercise trial decreased zonulin levels.

These reductions were measured by zonulin's presence in feces. Therefore, this reduction in zonulin suggest probiotic supplementation can improve intestinal barrier integrity while undergoing intensive exercise.

Ergogenic Effects. There have also been findings reporting a direct ergogenic benefit in exercise performance from probiotic supplementation [115, 116]. One study conducted by Shing et al. 2014 utilized trained male runners in a single group crossover design with a 28 day washout period and supplemented them for 4 weeks with 45 billion of a multi-strain probiotic supplement that included *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* strains. After 4 weeks of daily probiotic supplementation or placebo they completed a time-to-fatigue test in the heat. Shing et al. found when the runners were supplemented with the probiotic their run time-to-fatigue significantly increased with the average being $37:44 \pm 2:42$ vs $33:00 \pm 2:27$ with placebo. Serum LPS concentrations significantly increased between pre- and post-exercise, however, the probiotic supplementation was unable to significantly alter or reduce these increased concentrations. Furthermore, plasma concentrations of IL-6 and IL-10 were reported to also increase following the time-to-fatigue exercise test, however, similar to LPS the probiotic supplementation was unable to effect these increased secretions. Despite these findings, they are unclear on the exact mechanism(s) for this perceived direct exercise performance benefit from the probiotic supplementation.

Another study by Salarki et al. 2013 studied the effects of a probiotic yogurt on exercise performance in young adult female endurance swimmers [115]. The probiotic yogurt contained several probiotic strains with a total bacteria count of 40 Billion and was consumed for 8 weeks. Salarki et al. found the athletes supplemented with the probiotic yogurt resulted in a significant

improvement in VO_{2max} . They suggest this observed improvement in VO_{2max} was related to the reduction in the number of occurrences of respiratory infections as well as the probiotic yogurt fed athletes being healthier overall during the 8 weeks of training. Though limited, these two studies suggest probiotic supplementation can enhance exercise performance. However, it is unclear if these probiotic associated benefits are directly or indirectly related with the indirectly by way of enhancing immune function and reducing susceptibility to illness during chronic intensive exercise training thus allowing athlete to maximize training benefits.

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The Effects of Bifidobacterium Infantis 35624 on Exercise Performance, Inflammatory Markers, and Cognitive Outlook in Collegiate Female Swimmers

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Abstract

The purpose of this study was to determine the effects *Bifidobacterium infantis* 35624 (*B. infantis*35624) has on exercise performance, inflammation/immune function, and cognitive outlook during a six week exercise training phase in collegiate female swimmers. Using a two-group matched, double-blind, placebo-controlled design, seventeen NCAA Division 1 female swimmers were assigned to either group and supplemented daily for six weeks a 1×10^9 CFU dosage of *B. infantis*35624 (n=8) or placebo (n=9). Both groups underwent an intensive six week swim and resistance training program. Exercise testing (aerobic/anaerobic swim time trials and force plate vertical jump) as well as serum and salivary samples (cytokine and gastrointestinal (GI) inflammatory and immune markers) were collected pre, mid (week 3), and post (week 6). Recovery-stress questionnaire for athletes (RESTQ-Sport) was administered at baseline and at the end of each training week. Study data was analyzed by Analysis of Covariance (ANCOVA) by time point design with the respective baseline values of each dependent variable being the covariate. The *B. infantis*35624 group had a significant reduction in the anti-inflammatory marker IL-1ra ($p = 0.029$, $\eta_p^2 = .296$) and a noted statistical trend for a decrease in salivary IgA ($p = 0.060$, $\eta_p^2 = .231$) in comparison to placebo at mid-point. The *B. infantis*35624 group had significantly higher RESTQ-sport values in the sport recovery than the placebo group. *B. infantis*35624 in Division 1 female swimmers does not directly influence exercise performance, but did influence markers of inflammation/immune function as well as cognitive outlook during six weeks of exercise training.

Keywords: Probiotic, Swimmers, Exercise, Immune, Inflammation, Stress

Introduction

The human gut microbiota contains a diverse bacterial species understood to exert numerous physiological functions such as protection against pathogens, barrier effects, regulation of energy levels and metabolism, modulation of intestinal motility, and regulation of immunity within the entire gastrointestinal (GI) tract [1]. Probiotic bacteria are defined as live microorganisms that positively modulate microbiota and health of the host such as the human gut microbiota [2]. Probiotic supplementation has been demonstrated to beneficially impact the intestinal mucosal barrier and improve GI integrity thus, in turn, strengthening GI immune response, reducing mucosal inflammation, and decreasing oxidative stress [3-8].

Recent findings have shown a specific probiotic strain termed *Bifidobacterium infantis* 35624 (*B. infantis*35624), a subspecies of *Bifidobacterium*, to beneficially impact the immune response by inducing regulatory T cells (T_{reg}), which are designated to better contain reactions and limit the aggressive spread of immune responses to other tissues in human models within the gut as well as beyond the gut [9-11]. It has been previously reported that 6-8 weeks of *B. infantis* 35624 supplementation increases secretion of T_{reg} cells in the peripheral blood of healthy human subjects as well as reduce systemic circulation of pro-inflammatory biomarkers $TNF-\alpha$, IL-6 in clinical patients diagnosed with chronic fatigue syndrome (CFS) an immune-inflammatory disease [12] [9].

In addition to these systemic immunomodulatory effects, it has also been demonstrated that increased concentrations of *Bifidobacterium* within the gut, through supplementation, can positively affect emotional symptoms in CFS patients such as reducing levels of anxiety and improving mood [13]. Moreover, *B. infantis*35624 supplementation in the animal model has been found to positively influence neuronal systems and behaviors pertinent to depression [14].

These findings suggest *Bifidobacterium* and its subspecies *B. infantis* 35624 could play a role in gut-brain interactions and provide, through supplementation, a prospective therapeutic application for these probiotics in response to various forms of cognitive stress.

In sports and exercise, it is common for highly trained athletes such as elite swimmers to undergo periods (> 1 week) of intensive exercise training made up of repetitive, high volume, intense concentrated workloads combined with inadequate periods of physical rest [15]. This particular exercise training regimen has been reported to result in an increased susceptibility of upper respiratory tract infections, GI symptoms (i.e. nausea, cramps, vomiting), physical exhaustion (i.e. fatigue), cognitive stress, and overall reductions in sport performance [16-21]. These potential undesired outcomes during this intensive exercise training phase are hypothesized to occur from increased microtrauma (i.e. injury) to the muscle, GI tract, connective tissue, and joints [22, 23]. As a result, these injuries are proposed to initiate a local and “whole body” systemic inflammatory response that could attribute to a compromise in immune function, decreased exercise performance, and negatively alter mood states [22].

Thus, the systemic immunomodulatory and cognitive effects observed with *Bifidobacterium* and more importantly *B. infantis* 35624 supplementation in CFS patients presents an interesting finding for athletes like swimmers who participate in chronic, prolonged intensified exercise training resulting in potential changes to the GI tract, immune function, physical readiness, and cognitive stress. Therefore, the purpose of this novel study was to explore the effects of *B. infantis* 35624 supplementation in swimmers with respects to systemic inflammation, exercise performance, and cognitive stress during an intensive six week exercise training phase.

Methods

General Experimental Design. To determine the effects of *B. infantis* 5624 on systemic inflammatory response, exercise performance, and cognitive stress during an intensified exercise training phase, a double-blind, randomized placebo controlled Analysis of Covariance (ANCOVA) by time point design was utilized. The dependent variables for this study included markers of inflammation and immune function, multiple measures of exercise performance as well as a weekly assessment of cognitive stress-recovery. Participants who meet the inclusion criteria were randomly assigned, via stratified randomization, to ingest either *B. infantis* 35624 or placebo. Supplementation commenced at the onset of designated intensified training phase thus requiring baseline measurements to be completed at least 72 hours prior to beginning of the study and included all blood and salivary samples, exercise performance testing, and cognitive stress-recovery assessment. Additional blood and salivary samples and exercise testing to determine possible inflammation, immune, and performance variations during respective training phase was measured again at midpoint (week 3) and immediately upon completion (week 6) with the cognitive stress assessment being completed weekly (weeks 1-6).

Subjects. Twenty (n=20) Division 1 collegiate female swimmers were recruited to participate in this study. All inclusion/exclusion criteria followed the Groeger et al. 2013 study using *B. infantis* 35624 supplementation in CFS patients which states any participants pregnant or diagnosed with a lactose intolerance and immunodeficiency in addition to any previous abdominal surgery (with the exception of hernia repair or appendectomy) or reported psychiatric illness were excluded from the study [9]. Also, all recruited participants were required to refrain from taking any anti-inflammatory medications or antibiotics for the previous month as well as throughout the duration of the six week study. Finally, all participants must also report not

having any nutritional supplements (with the exception of a multi-vitamin, vitamin C, vitamin D, and iron supplements) and/or ergogenic aids for the preceding one week period. All participants were informed of the study procedures and required to provide a written consent. The study was approved through the University's Human Research Protection Program and Institutional Review Board.

Nutrition and Supplementation. One week prior to beginning supplementation and training, all participants received sport-specific nutrition education by a Registered Dietitian (RD) who is a Certified Specialist in Sports Dietetics (CSSD) addressing appropriate nutritional needs throughout duration of the study. Participants then completed a three day dietary food log prior to start of the study followed by two additional three day dietary food logs at midpoint and immediately upon completion of study. These recorded dietary food logs were analyzed to ensure nutritional habits remained similar as well as each log refraining from foods rich in probiotics (ex. Kefir) and caffeine which could potentially influence our findings during the course of supplementation and exercise training.

Subjects began the oral encapsulated supplementation regime of either 1 pill of 4mg (1×10^9 colony forming units (CFU) live bacteria) B.infantis35624 or an identical encapsulated placebo pill consisting of maltodextrin daily throughout their designated six week training phase. The B.infantis 35624 supplement is commercially available for purchase and the supplement provided to the probiotic subject group was obtained from a local retail store. The viability of the B.infantis 35624 commercial product has been independently tested by ConsumerLabs.com verifying the listed amount of probiotic organisms in each capsule does indeed contain 1×10^9 CFU of live bacteria. The entire respective supplementation dosage was ingested once each day. To ensure compliance, subjects were blindly provided the supplement each day of practice

and were visually observed ingesting their respective supplement by a member of the investigative team followed by notation verifying ingestion took place. On the day(s) participants were not required to train each participant received an individual supplement container containing their respective pill(s) and instructed to take as directed and return the empty container as well as complete a weekly supplement adherence verification form to help ensure compliance.

Intensified Exercise Training Phase. The designated six week intensified exercise training phase was similar for each supplemented group and was coordinated/conducted by the subject's team sport coach and strength & conditioning coach. Both respective coaches coordinated and conducted the subject's exercise training program during the entire competitive season prior to onset of the study. All subjects received two weeks of physical recovery before the start of the study's training phase which occurred during team's off-season. Weeks 1-4 of the intensified exercise training included '8' hours of swim training in addition to two – 30 minutes resistance training sessions with the accumulative swim practice volume totaling approximately 20 kilometers each week. Weeks 5-6 the exercise training increased to '20' hours of swim training which included within the '20' hrs three– 45 minutes of resistance training sessions with the accumulative swim practice volume totaling approximately 40 kilometers each week.

General Performance Testing. Subjects underwent exercise performance testing at three separate times points (pre, mid, and post) during the designated supplementation and training phase. Testing included a vertical jump test as well as an aerobic and anaerobic swim-specific performance assessment. The vertical jump on a force plate (9260AA6; Kistler Instruments, Winterthur, Switzerland) using custom-designed software (SpartaTrac; SPARTA Performance Science) was completed for all subjects in the morning before the team's resistance training

session in accordance with a standing vertical jump movement to measure rate of eccentric force production ($\text{N}\cdot\text{s}^{-1}$), concentric force production ($\text{N}\cdot\text{kg}^{-1}$), and overall vertical jump height (meters) per the team's specific force plate jump protocol.

Overall vertical jump height (meters) was calculated using the impulse-momentum relationship ($\mathbf{J} = \int \mathbf{F} dt = \Delta \mathbf{p}$) where the difference in impulse (\mathbf{J}) of ground reaction force (JGFR) and the impulse of the jumper's body weight (JBW) determines subject's jump height [24]. Eccentric force production ($\text{N}\cdot\text{s}^{-1}$) was calculated between the points at which vertical ground reaction force exceeds body mass during the countermovement on through to the point of minimum displacement of the countermovement with the reported mean eccentric force production calculated between the minimum and maximum force during the eccentric phase [25]. Concentric force production ($\text{N}\cdot\text{kg}^{-1}$) was calculated 0.001 seconds after the end of the eccentric phase to the point of take-off with the reported mean concentric force production determined as the average force achieved during the respective phase in relation to body mass ($\text{N}\cdot\text{kg}^{-1}$).

The aerobic swim test was a 500 meter freestyle time trial and the anaerobic swim test was a 100 meter freestyle time trial. Each swim test was conducted in a 25 meter training pool located in team's home natatorium. Both swim tests were standard assessments of exercise performance frequently conducted by head coach during the competitive season.

Blood and Salivary Collection, Systemic and GI Inflammatory/Immune Markers. In accordance with the supplementation and exercise performance testing, blood and salivary samples were also obtained pre, mid, and post of the supplementation/intensified training experiment. These samples were collected at rest before the exercise training session as well as at the same day and time for each measurement time point. At each respective time point 5mL

of blood was collected from each subject via venous puncture in the afternoon, at rest, and before afternoon exercise training occurred that day. To obtain serum samples, all collected blood was allowed to clot at room temperature then separated by refrigerated centrifugation and subsequently aliquoted and frozen at -80°C for later analyses according to assay procedure requirements. These frozen serum samples were used to measure markers of systemic inflammation (IFN- γ , IL-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-17F, IL-22, TNF- α) using a custom luminex magnetic bead-based human cytokine panels A&B (R&D Systems, Bio-Techne) as well as markers of GI integrity (endotoxin/LPS and LPS Binding Protein (LBP)) using a pierce LAL chromogenic endotoxin quantitation kit (Thermo Scientific) and human LBP duoset ELISA (R&D Systems, Bio-Techne).

Salivary samples using Salimetrics SalivaBio swabs were used to collect and measure immunoglobulin A (IgA) immediately following collection of blood/serum samples. However, before subjects provided a blood sample, per Salimetrics instructions, they were required to thoroughly rinse out their mouth with tap water and wait 10 minutes before providing a salivary sample. Once ready to provide a sample, subjects were instructed to remove the SalivaBio Oral Swab (SOS) from packaging and place, without touching, in their mouth and under the tongue. Subjects were required to keep the SOS under their tongue for two minutes to ensure it is fully saturated. After two minutes and full saturation, subjects placed the SOS, by mouth and without touching, into the swab storage basket insert located in the swab storage tube which was then capped and frozen immediately at -80°C to be later analyzed using a Salimetrics salivary secretory IgA indirect enzyme immunoassay kit.

Cognitive Stress-Recovery Assessment. A cognitive stress-recovery assessment using the Recovery-Stress Questionnaire for Athletes was utilized at the end of each training week to

determine the participant's cognitive outlook during the intensified training study [21]. The RESTQ-Sport questionnaire utilized was the short version consisting of 52 items used to assess, in a multidimensional way, an athlete's stress-recovery state during a specific training cycle. Each item answered in the RESTQ-52 Sport, which is based on a Likert-type scale with values ranging from 0 (never) to 6 (always), indicates how often an athlete participated in certain activities during the previous three days and nights. Once completed each week, these 52 items generate 19 specific stress-recovery scales which are grouped into four categories; general stress, general recovery, sport stress, and sport recovery in efforts to reflect, quantitatively, each supplementation group's subjective cognitive assessment of stress and recovery during the intensified exercise training load.

Statistical Analyses. All statistical analyses was performed using SPSS V.22 (Chicago, IL) software. Study data was analyzed by Analysis of Covariance (ANCOVA) by time point design with the respective baseline values of each dependent variable being the covariate. The dependent variables were defined as the blood and salivary inflammatory/immune markers, exercise performance tests, and RESTQ-52 Sport scale scores while the independent variables were the supplemented vs non-supplemented groups and time. Data was considered statistically significant when the probability of type I error is ≤ 0.05 . Dietary food logs were analyzed using a One-Way ANOVA. To determine statistical significance of ≤ 0.05 between groups a robust test of equality of means using Welch/Brown-Forsythe statistics was utilized due to homogeneity of variances being violated because sample sizes were different among the groups. In addition, due to unequal variances Dunnett's C post hoc analysis was used.

Results

Study population, supplementation, and nutrition. Seventeen of the twenty recruited subjects completed the study. Two subjects dropped out due to illness while the third subject dropped out due to quitting the team. Therefore, nine subjects in the placebo group and eight subjects in the *B. infantis* 35624 group completed the six week intensified exercise training study.

Supplementation compliance throughout the study, which included weekends, was 96.94% in the placebo group and 97.81% in the *B. infantis* 35624 group. Dietary food logs reported no statistical difference in total energy calories, carbohydrates, protein, and fat dietary intake between the groups throughout the study.

Swim and force plate performance testing. Table 1 details the no statistical significant difference observed between supplemented groups aerobic (week 3, $p = .485$) (week 6, $p = .762$), and anaerobic swim (week 3, $p = .700$) (week 6, $p = .735$) performance testing as well as their force plate power testing measuring eccentric force production (week 3, $p = .666$) (week 6, $p = .961$), concentric force production (week 3, $p = .613$) (week 6, $p = .606$), and overall vertical jump height (week 3, $p = .280$) (week 6, $p = .192$).

Systemic cytokine, GI, and Salivary IgA markers. Systemic inflammatory markers IFN- γ , IL-1b, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-17F, IL-22, TNF- α were below detectable levels and was unable to obtain any values. IL-1ra in the *B. infantis* 35624 group had statistically significantly lower ($p = 0.029$, $\eta_p^2 = .296$) serum levels at mid-training (week 3) in comparison to the placebo group (Figure 1). However, no statistical significant difference between groups in IL-1ra levels at post-training (week 6, $p = .769$) was observed. Serum GI markers endotoxin (LPS) and LBP were also not statistically significantly different between the supplementation groups. A trend towards statistical significance was observed in Salivary IgA

levels with *B. infantis* 35624 group measuring lower ($p = 0.060$, $\eta_p^2 = .231$) at mid-training in comparison to the placebo group (Figure 2). However, similar to IL-1ra levels, no significant difference was found between groups at post-training (week 6, $p = .436$).

RESTQ-52 Sport. Table 3 shows the summary scores in each stress-recovery scale of the RESTQ-52 Sport for all seven measurements (weeks 0-6) taken during the study. During the first four weeks, which encompassed the ‘8’hr training schedule, no statistical significant difference was observed between the groups until weeks 3 and 4 with significant changes noted within the “General Stress” category under the *Conflicts/pressure* scale and *Social stress* scale respectively. The *B. infantis* 35624 supplemented group reported during week 3 of training significantly higher values of *Conflicts/pressure* in comparison to placebo group (probiotic – $2.08 \pm .97$, placebo – 1.67 ± 1.12). Following the conclusion of week 4, *B. infantis* 35624 group had significantly lower values in *Social stress* in comparison to placebo group (probiotic – $1.00 \pm .65$, placebo – $1.72 \pm .51$). During the last two weeks of the study, which required both groups to undergo the ‘20’hr exercise training regime, a statistical significant difference was found within the “Sport Recovery” category under the *Personal accomplishment* scale (week 5) and *Self-regulation* scale (weeks 5&6). The *B. infantis* 35624 group recorded significantly higher values in both the *Personal accomplishment* (probiotic – $3.43 \pm .93$, placebo – $2.75 \pm .87$) and *Self-regulation* (Figure 4) in comparison to placebo group.

Discussion

To the authors’ knowledge, this was the first study to investigate *B. infantis* 35624 supplementation in healthy collegiate athletes. More specifically, to study the effects *B. infantis* 35624 supplementation has on exercise performance, inflammation/immune function, and cognitive stress-recovery outlook in Division 1 collegiate female swimmers during a six week

intensified exercise training phase. We did not observe any exercise performance differences between the supplemented group's aerobic and anaerobic swim time trials as well as their force plate vertical jumps throughout the study. Similar to our findings, previous studies have also been unable to demonstrate probiotic supplementation's ability to directly affect exercise performance supplementing other probiotic bacteria strains and not *B. infantis* 35624 [19]. Rather, its effects on exercise performance has been suggested to be more indirect as reported by Salarkia et al. 2013 using probiotic yogurt in young adult female endurance swimmers [26]. The probiotic yogurt contained several probiotic strains (*Lactobacillus Acidophilus*, *Lactobacillus Delbrueckii Bulgaricus*, *Bifidobacterium Bifidum*, and *Streptococcus Salivarius Thermophilus*) with a total bacteria count of 4×10^{10} CFU and was consumed for 8 weeks. They found swimmers supplemented with the probiotic yogurt had significant improvements in VO_{2max} . These VO_{2max} improvements were suggested to be related to reductions in the number of occurrences of respiratory infections in addition to the probiotic yogurt supplemented athletes being healthier overall during the eight weeks of training. As a result of the yogurt group reporting an enhanced immune function and a reduced susceptibility to illness they suggest the probiotics indirectly allowed the athletes to maximize their training benefits during the eight weeks of intensive exercise training resulting in a significant increase in VO_{2max} .

In the present study, all subjects who completed the six week exercise training were not diagnosed, by the team's physician or physician's assistant, with any respiratory infections or illnesses that kept them out from participation in the study. The two subjects diagnosed with an illness and unable to train for more than several days were removed from the study and not included in the analysis. Since we were unable to find any differences in our exercise performance testing throughout the study and did not observe any changes between the groups'

occurrences of respiratory infections, or even trends reflecting the probiotic group to be less susceptible to illness, our findings were unable to support a potential indirect effect on exercise performance from probiotic supplementation as suggested by Salarkia et al. 2013.

According to the results of our study only a significant decrease in the systemic cytokine marker IL-1ra within the probiotic group at mid-training was found. IL-1ra is an anti-inflammatory cytokine and is a natural antagonist that competes with IL-1 β , a pro-inflammatory cytokine, for receptor binding without initiating a pro-inflammatory signal transduction [27, 28]. Significant elevations in IL-1ra levels of up to 40 fold increases have been reported to occur following most endurance exercise [29]. Research suggests this increase following endurance exercise is in response to the rapid increase of systemic IL-6 concentrations [30, 31]. Since IL-1ra originates from mononuclear and polymorphonuclear leukocytes as opposed to IL-6 which is produced locally from the working muscle during exercise, it is believed the leukocyte derived IL-1ra production is significantly increased when the pro-inflammatory production of IL-6 within skeletal muscle gets released in efforts to exert strong anti-inflammatory effects [31-35]. Thus, it is suggested the levels of systemic IL-1ra reflect the production of IL-6 [36]. Unfortunately, we were unable to detect and measure systemic levels of IL-6. Therefore, we are unclear as to why IL-1ra levels during the mid-point of training were lower than placebo. However, given previous findings by Groeger et al. 2013 demonstrating reduced systemic levels of IL-6 in CFS patients with B. infantis 35624 supplementation it could be speculated similar effects occurred in our probiotic supplemented group's systemic levels of IL-6 during the '8'hr exercise training regimen [9]. As a result of these "theorized" lowered IL-6 levels during and following exercise, it is plausible IL-1ra followed a similar pattern with systemic levels being lower after exercise and at rest in comparison to the placebo group.

As previously stated, we were unable to detect or even extrapolate the additional cytokines and their levels despite obtaining a high bead count for each marker during analysis. A previous study has reported IL-1b and IL-5 to be below detectable limits in the serum of healthy subjects [37]. However, cytokines IFN- γ , IL-2, IL-4, IL-6, IL-10, IL-13, IL-17, IL-17F, TNF- α have been found to be detectable with referenced normative levels in the serum of healthy subjects [37]. Therefore, our inability to detect these previously referenced cytokines is possibly due to the sensitivity of our antibodies, and the minimum detectable levels using our system with resting (i.e. outside of physical exercise) serum cytokine levels in healthy subjects such as highly trained collegiate athletes.

To the author's knowledge, this is the first study to examine the effects of probiotic supplementation on salivary IgA in swimmers during an intensive exercise training phase. A statistical trend, non-significant, reduction in salivary IgA at mid-point of the study was noted in the probiotic group. IgA is the predominant protein in the mucosal antibody response which is critical in helping neutralize toxins and remove pathogens [38]. Previous studies have shown chronic, prolonged intensive exercise to decrease salivary IgA levels and subsequently increase likelihood of developing an upper respiratory tract infection in athletes [39, 40]. This inverse relationship between salivary IgA and development of upper respiratory tract infection has been reported to occur in marathon runners [41] with salivary IgA secretion being reduced by 10% followed by 25% of the runners reporting an upper respiratory tract infection within two weeks after completing the race. As previously stated, there were no respiratory infections or illnesses amongst any of our subjects which included all subjects in the probiotic group. Despite a nearly 33% reduction in salivary IgA secretion at mid-point in comparison to baseline within the probiotic group as well as a noted statistical trend difference in comparison to placebo it did not

appear to negatively affect the subject's immune function and ability complete the required exercise training.

Gut inflammatory markers endotoxin (LPS) and LBP measured no significant difference between the groups throughout the study. To our knowledge this is the first time both GI markers have been measured in swimmers undergoing an intensive exercise training phase. Our endotoxin levels measured amongst our group of collegiate swimmers were similar to levels reported by Lira et al. 2010 in highly trained cyclists [42]. Lira et al. 2010 found and concluded that endotoxin levels correlate negatively with highly trained subjects. Since our subjects are also highly trained we observed a similar correlation despite exercise training being strenuous and intensive. As a result of our endotoxin findings it was not surprising for LBP levels to remain unchanged between both the probiotic and placebo groups. LBP has been previously shown to play an important role in the inflammatory response that is secondary to increased blood levels of endotoxin [43, 44]. Since we observed no change, or difference, in serum endotoxin levels between groups throughout the study then it would almost expected for LBP levels to follow a similar pattern.

Our cognitive RESTQ52-Sport findings suggest the '8' hr exercise training regime (weeks 1-4) was not a high enough exercise training load to illicit changes within the reported scales of general stress, general recovery, sport stress, and sport recovery. During those respective weeks we only found a couple of single time points (i.e. one week) differences between the two supplemented groups and lacked any repeated weekly differences to more clearly indicate a potential stress-recovery effect from both the training load and/or supplementation. A similar finding in regards to training load in swimmers using the RESTQ-Sport has been reported [20]. Gonzalez-Boto et al. 2008 also did not observe any differences

within the swimmers stress-recovery scales during exercise training phases that involved overall swim distances ranging between 3.2km – 3.9km and total swim durations of each session between 87 – 102 minutes. Interestingly, our ‘8’ hr exercise training week averaged a daily overall swim distance of 4km and swim duration of 95 minutes. Therefore, it appears our weeks 1-4 exercise training were similar to Gonzalez-Boto et al. 2008 which supports our similar lack in findings. When our subjects transitioned to the ‘20’hr week exercise training phase (weeks 5&6) which included daily overall swim distance of 6.67km and swim duration of 135 minutes we observed the probiotic group to have significantly higher values in both respective weeks under the scale of self-regulation which is part of the sport recovery group. The self-regulation scale is defined as the use of mental skills for athletes to prepare, push, motivate, and set goals for themselves [21]. Therefore, while undergoing a significantly higher exercise training load this cognitive scale suggest the probiotic supplemented group felt they were able to mentally push and motivate themselves more than the placebo group. Gonzalez-Boto et al. 2008 reported significant reductions in their swimmer’s sport recovery (being in shape, self-efficacy), sport stress (emotional exhaustion, injury), and general recovery (physical recovery) when overall daily swim distance 5km and overall swim duration was 137 minutes. Therefore, this finding suggests that if we would have continued to supplement *B. infantis* 35624 for additional weeks (ex. 4-6 weeks) while undergoing this high volume ‘20’ hr exercise training load would have it been possible for the probiotic group to maintain a higher level of self-regulation resulting in an indirect means of enhancing exercise performance based simply upon the probiotic group’s improved balance between cognitive stress-recovery outlook.

Conclusion

This was the first study to investigate the effects of *B. infantis* 35624 probiotic supplementation in athletes such as collegiate female swimmers. Our findings indicated that daily *B. infantis* 35624 supplementation during a six week exercise training phase did not directly influence exercise swim performance. However, the probiotic group reported a reduction in IL-1ra and noted trend reduction in salivary IgA at mid-point as well as higher reports of sport recovery during two weeks of the higher training load. These findings suggest a potential chance for an indirect effect on exercise performance if supplementation duration was extended and *B. infantis* 35624 dosage altered. In regards to dosage, the Groeger et al. 2013 study provided for its CFS patients a *B. infantis* 35624 daily dosage of 1×10^{10} CFU for 6-8 weeks and reported significant systemic reductions in IL-6 and TNF- α [9]. Our daily dosage was 1/10 of the Groeger et al. 2013 study at 1×10^9 CFU. Therefore, it would be of interest to investigate if a higher dosage in an athletic population could have a more profound impact on their systemic inflammation and immune function continually throughout a higher training load which could as a result, in theory, provide an indirect exercise performance benefit as suggested by Salarkia et al. 2013. Furthermore, it appears the higher exercise training loads observed in weeks 5 & 6 of our study was similar to the high volume training phase previously discussed by Gonzalez-Boto et al. 2013 study. Gonzalez-Boto et al. reported numerous undesired changes in their swimmers RESTQ-Sport stress recovery scales where our probiotic group during a similar high training load reported weekly greater values in self-regulation/sport recovery than the placebo group which was significantly lower. Therefore, it would be of further interest to provide *B. infantis* 35624 supplementation for a more extended period of time while these swimmers are undergoing more chronic/weekly higher training loads to observe if these initial findings would continue as

well as potentially positively influence other stress-recovery categories. In theory these probiotic derived positive cognitive changes could also indirectly enhance exercise performance due to a better balance within an athlete's stress-recovery state. Therefore, additional research is recommended to further investigate the duration and dosage questions in hopes of better understanding the potential indirect effects *B. infantis* 35624 supplementation might have on the health and performance of athletes.

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Table 1 Exercise Performance Testing

	Week 0	Week 3		Week 6	
	Baseline	Probiotic	Placebo	Probiotic	Placebo
	(n=17)	(n=8)	(n=9)	(n=8)	(n=9)
Aerobic Swim (seconds)	452.5 ± 20	443.2 ± 10.2	439.6 ± 10.2	440.8 ± 9.3	439.3 ± 9.3
Anaerobic Swim (seconds)	62.9 ± 2.7	62.2 ± .95	61.9 ± .92	62.3 ± 1.45	62.7 ± 1.23
Eccentric Force (N·s⁻¹)	3244 ± 1598	3202 ± 270	2960 ± 253	2897 ± 310	2872 ± 271
Concentric Force (N·kg⁻¹)	17.7 ± 1.5	17.5 ± 7.8	17.3 ± 7.6	17.4 ± 5.6	17.2 ± 5.4
Vertical Jump Height (m)	.34 ± .07	.35 ± .01	.32 ± .01	.35 ± .01	.32 ± .01

Exercise performance testing week 0 baseline covariate values corresponding to the week 3 and week 6 measurements taken during the training period.

Values are mean ± SD.

Table 2 RESTQ52-Sport cognitive stress-recovery

	Week 0		Week 3		Week 4		Week 5		Week 6	
Scale	Probiotic	Placebo	Probiotic	Placebo	Probiotic	Placebo	Probiotic	Placebo	Probiotic	Placebo
General Stress										
1. General Stress	1.71 ± .48	1.61 ± .82	1.71 ± 1.1	1.72 ± .90	.93 ± .67	1.28 ± .76	1.64 ± 1.3	1.94 ± 1.4	2.29 ± .99	2.44 ± 1.1
2. Emotional Stress	1.58 ± .58	1.78 ± .67	1.83 ± .93	2.00 ± 1.1	1.08 ± .66	1.78 ± .62	1.75 ± .94	2.1 ± 1.11	2.5 ± 1.1	2.44 ± .68
3. Social Stress	1.50 ± .87	1.61 ± .55	1.64 ± .85	1.67 ± .61	*1.00 ± .65	1.72 ± .51	1.86 ± .99	2.00 ± 1.2	2.43 ± 1.1	2.33 ± 1.0
4. Conflicts/pressure	1.75 ± .82	2.44 ± 1.1	*2.08 ± .97	1.67 ± 1.1	1.75 ± .99	2.05 ± 1.3	2.25 ± 1.4	2.22 ± 1.0	3.00 ± 1.1	2.56 ± 1.3
5. Fatigue	2.00 ± 1.0	1.89 ± 1.3	2.17 ± .93	1.94 ± 1.4	1.42 ± 1.7	1.72 ± .44	2.25 ± 1.7	2.33 ± .75	3.33 ± 1.6	2.44 ± 1.1
6. Lack of energy	1.92 ± .92	2.22 ± .91	2.50 ± 1.2	1.67 ± .90	1.58 ± 1.1	1.83 ± .50	1.25 ± 1.0	1.78 ± .83	2.25 ± 1.2	2.28 ± .87
7. Physical complaints	0.75 ± .52	1.39 ± .60	1.42 ± 1.1	1.56 ± .53	0.83 ± .82	1.22 ± .67	1.67 ± 1.3	1.50 ± .79	2.00 ± .89	1.55 ± .77
General Recovery										
8. Success	2.00 ± .63	3.28 ± .83	2.17 ± .75	2.61 ± .74	2.67 ± .93	3.17 ± 1.1	3.25 ± 1.5	2.78 ± 1.1	2.75 ± 1.1	3.11 ± 1.1
9. Social recovery	3.42 ± .66	4.28 ± .67	2.67 ± .93	3.67 ± 1.0	3.50 ± .32	3.89 ± .70	3.50 ± .89	3.56 ± 1.2	3.33 ± 1.2	3.50 ± 1.3
10. Physical recovery	1.67 ± .82	2.44 ± 1.0	2.00 ± .77	2.33 ± .79	2.42 ± .97	2.61 ± .89	2.75 ± 1.2	2.50 ± .75	1.83 ± .93	2.39 ± .82
11. General well-being	2.75 ± 1.3	3.94 ± .68	2.83 ± 1.6	3.17 ± 1.2	3.75 ± .42	3.67 ± .97	3.00 ± 1.4	3.22 ± 1.1	2.83 ± 1.1	3.17 ± 1.3
12. Sleep quality	3.50 ± 1.3	3.78 ± .67	3.86 ± .56	3.89 ± .65	4.07 ± .89	3.72 ± .75	4.14 ± .75	3.67 ± .79	3.36 ± .99	3.50 ± 1.0
Sport Stress										
13. Disturbed breaks	0.96 ± .39	1.25 ± .80	1.04 ± .57	1.25 ± .73	1.04 ± .83	1.25 ± .72	1.07 ± .66	1.56 ± .60	2.04 ± 1.3	1.86 ± 1.1
14. Emotional exhaustion	1.64 ± .91	1.75 ± .94	1.64 ± .35	1.78 ± 1.3	1.14 ± .75	1.47 ± 1.1	1.69 ± 1.3	1.81 ± .93	1.68 ± 1.3	2.25 ± .93
15. Injury	2.32 ± .88	1.86 ± 1.2	2.89 ± .93	2.56 ± 1.4	2.14 ± .75	2.19 ± 1.1	3.04 ± 1.2	2.64 ± .82	3.29 ± 1.2	2.19 ± .87
Sport Recovery										
16. Being in shape	1.75 ± 1.1	2.58 ± .94	2.14 ± .63	2.92 ± 1.4	2.75 ± .60	3.39 ± 1.0	2.68 ± 1.1	2.92 ± .94	2.54 ± 1.1	2.78 ± 1.1
17. Personal accomplishment	2.54 ± 1.0	3.14 ± .49	2.57 ± 1.1	3.31 ± .99	3.32 ± .93	3.14 ± .95	*3.43 ± .93	2.75 ± .87	2.79 ± 1.2	2.72 ± 1.1
18. Self-efficacy	1.89 ± 1.1	2.64 ± .87	2.43 ± .70	3.47 ± 1.1	3.07 ± .79	3.44 ± .92	3.25 ± 1.1	3.00 ± 1.0	3.08 ± 1.2	3.08 ± 1.2
19. Self-regulation	2.35 ± 1.3	3.03 ± .81	2.68 ± 1.0	3.17 ± .98	3.11 ± 1.2	3.17 ± 1.1	*3.54 ± 1.1	3.11 ± .94	*3.46 ± .92	2.89 ± 1.0

Scores in the different scales of the RESTQ52-Sport corresponding to the seven measures (Week 0-6) taken during the training period.

Values are mean ± SD. * $p \leq 0.05$ significant difference between groups each week.

Figure 1 Serum concentrations of IL-1ra in collegiate female swimmers pre, mid, and post 6 weeks of exercise training and treatment. Probiotic the B. infantis35624 supplemented group, Placebo group, PRE week 0, MID week 3, POST week 6; n = 8 (B. infantis35624 supplementation), n = 9 (placebo). Values are means \pm SD. There was a significant difference between groups at MID of exercise training and treatment: * P <0.05 (ANCOVA).

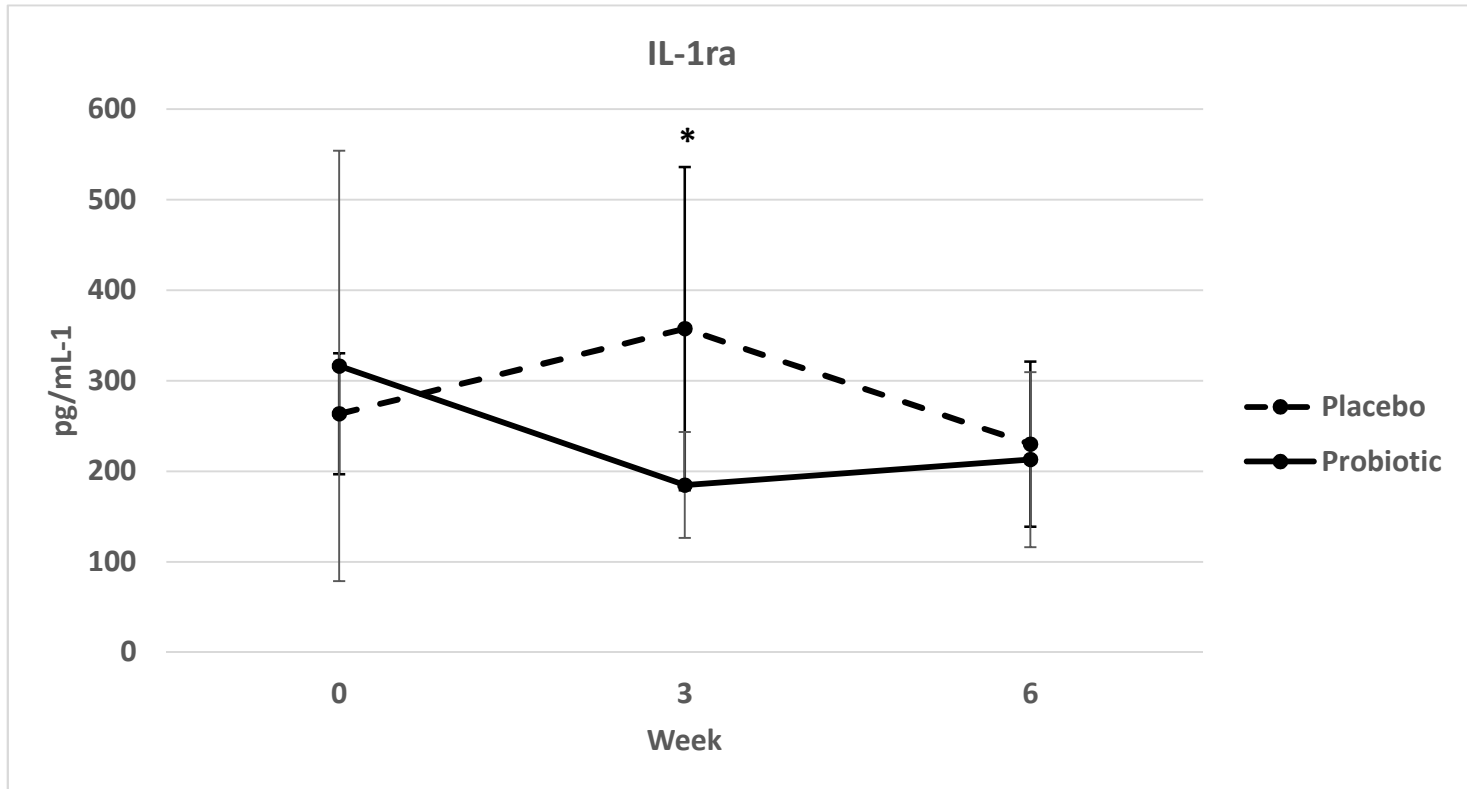


Figure 2 Salivary concentrations of IgA in collegiate female swimmers pre, mid, and post 6 weeks of exercise training and treatment. *Probiotic* the B. infantis35624 supplemented group, *Placebo* group, *PRE* week 0, *MID* week 3, *POST* week 6; n = 8 (B. infantis35624 supplementation), n = 9 (placebo). Values are means \pm SD. There was a trend towards significant difference between groups at MID of exercise training and treatment: \dagger P = 0.06 (ANCOVA).

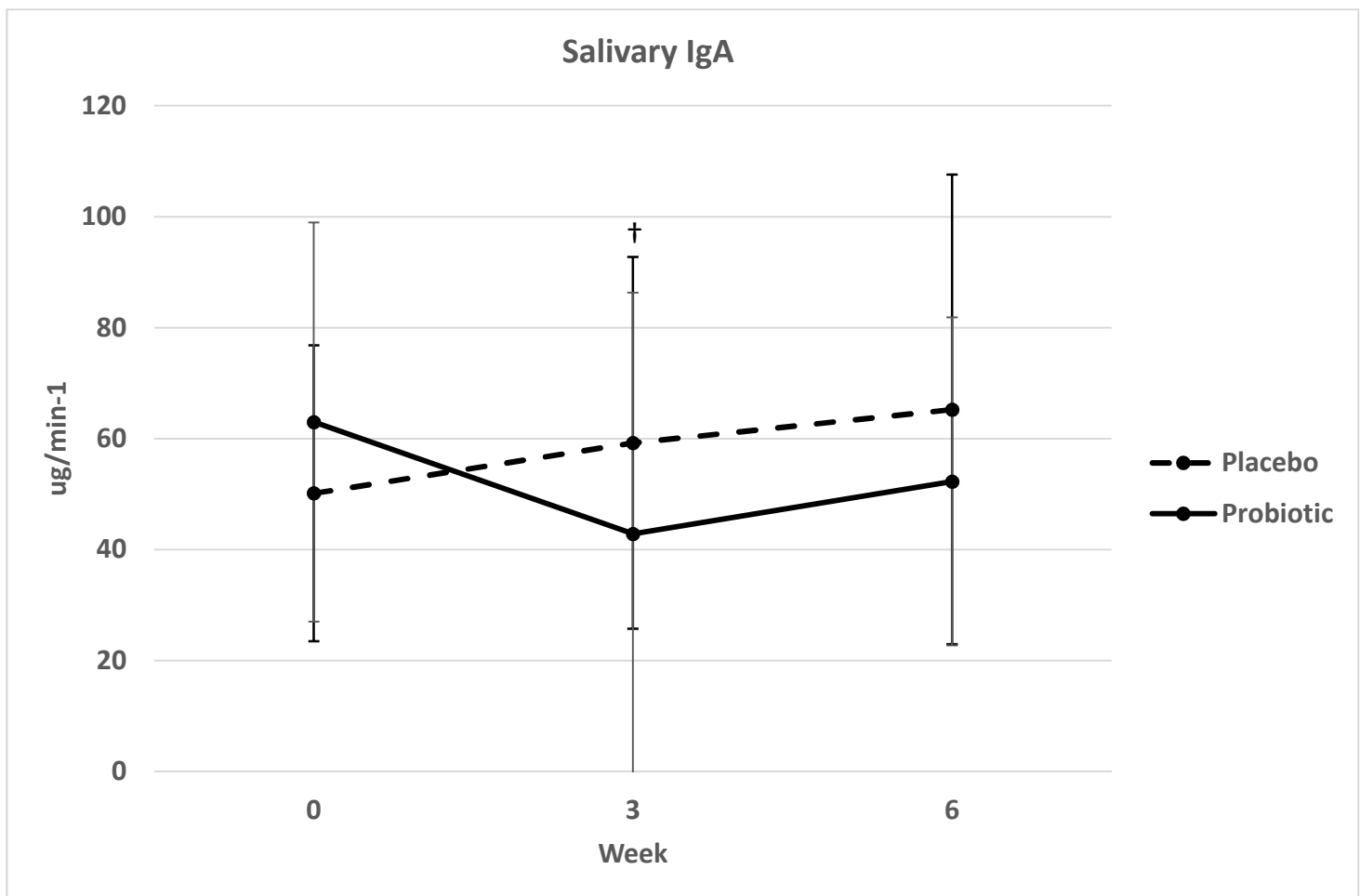
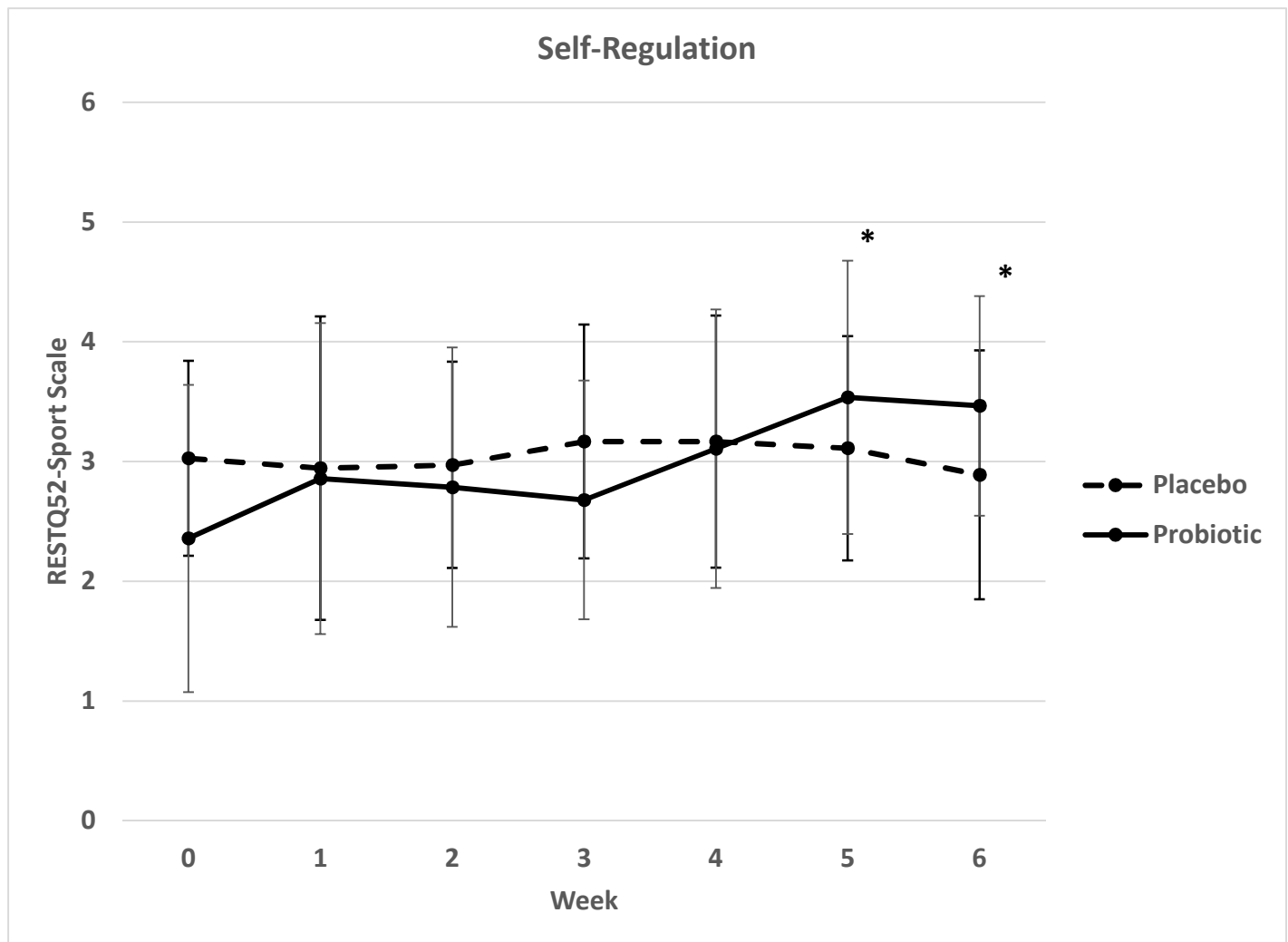


Figure 3 RESTQ52-Sport weekly self-regulation scores in collegiate female swimmers in 6 weeks of exercise training and treatment. The use of mental skills for athletes to prepare, push, motivate, and set goals for themselves are assessed by this scale. RESTQ52-Sport scale is defined as 0-never, 1-seldom, 2-sometimes, 3-often, 4-more often, 5-very often, 6-always. *Probiotic* the *B. infantis*35624 supplemented group, *Placebo* group, Week 0-6; n = 7 (*B. infantis*35624 supplementation), n = 9 (placebo). Values are means \pm SD. There was a significant difference between groups at Week 5 and Week 6 of exercise training and treatment: * P <0.05 (ANCOVA).



Appendix A
IRB#, Approval Letter

APPROVAL OF PROTOCOL

March 4, 2016

Andrew Fry acfry@ku.edu

Dear Andrew Fry:

On 3/4/2016, the IRB reviewed the following submission:

Type of Review:	Initial Study
Title of Study:	The Effects of Bifidobacterium Infantis 35624 on Inflammatory Markers and Exercise Performance in Collegiate Female Swimmers
Investigator:	Andrew Fry
IRB ID:	STUDY00003296
Funding:	Name: Procter & Gamble Co.
Grant ID:	
Documents Reviewed:	<ul style="list-style-type: none"> • HSCL_Revised Signed_Consent_Form_Probiotic_Exercise_Revision 3_ Clean Version.docx, • HSCL_Revised Signed_Consent_Form_Probiotic_Exercise_with Tracked Changes.v.9.docx, • Correspondence_for_STUDY00003296.doc, • HSES APL BBP approval 020220151 (2).pdf, • B infantis 35624 and Exercise_Revised Submission Form.v.8_ Includes EHS Clarifications.pdf, • Food Diary.pdf, • KU Health Exercise Status Questionnaire_Probiotic.v.1.doc, • RESTQ-52 Sport.pdf

The IRB approved the submission from 3/3/2016 to 3/2/2017.

1. Before 3/2/2017 submit a Continuing Review request and required attachments to request continuing approval or closure.
2. Any significant change to the protocol requires a modification approval prior to altering the project.
3. Notify HSCL about any new investigators not named in original application. Note that new investigators must take the online tutorial at https://rgs.drupal.ku.edu/human_subjects_compliance_training.
4. Any injury to a subject because of the research procedure must be reported immediately.
5. When signed consent documents are required, the primary investigator must retain the signed consent documents for at least three years past completion of the research activity.



If continuing review approval is not granted before the expiration date of 3/2/2017 approval of this protocol expires on that date.

Please note university data security and handling requirements for your project:
<https://documents.ku.edu/policies/IT/DataClassificationandHandlingProceduresGuide.htm>

You must use the final, watermarked version of the consent form, available under the “Documents” tab in eCompliance.

Sincerely,

Stephanie Dyson Elms, MPA

IRB Administrator, KU Lawrence Campus

Appendix B
Informed Consent

Informed Consent Statement/HIPAA Authorization

The Effects of Bifidobacterium Infantis 35624 on Inflammatory Markers and Exercise Performance in Collegiate Female Swimmers

INTRODUCTION

Kansas Athletics, Inc. and The Department of Health, Sport, and Exercise Sciences at the University of Kansas supports the practice of protection for human subjects participating in research. The following information is provided for you to decide whether you wish to participate in the present study. You may refuse to sign this form and not participate in this study. You should be aware that even if you agree to participate, you are free to withdraw at any time. If you do withdraw from this study, it will not affect your relationship with this unit, the services it may provide to you, or the University of Kansas.

PURPOSE OF THE STUDY

The purpose of this novel study is to examine in a double blind, placebo controlled design, whether B.infantis 35624 probiotic supplementation for six weeks in collegiate female swimmers will significantly affect the inflammatory/immune response during an intensified training load subsequently enhancing sport performance and cognitive outlook. It is hypothesized B.infantis 35624 supplementation will influence the inflammatory response during an intensified training phase and enhance exercise performance as well as improve cognitive outlook.

B.infantis 35624 Probiotic Supplement

The B.infantis 35624 supplement product is named Align. The Align product has been commercially available for years to the general public. It is not a drug. It contains only one ingredient which is the B. infantis 35624 probiotic strain. This specific probiotic strain is naturally found in healthy human gastrointestinal tissue which is how it was first discovered 20 years ago. Therefore, this supplement does not result in ingesting a foreign substance. No adverse effects have ever been reported in any previous peer-reviewed literature using this particular supplement. The dose described below is the standard recommended dose by Proctor & Gamble per supplement facts label. Funding for this study is being provided by Proctor & Gamble. Proctor & Gamble is the company that makes and manufactures the Align supplement product.

PROCEDURES

Regardless of your participation status in this research study you will be required to participate in a six week intensified exercise training regime which will include exercise training in the Robinson Natatorium as well as in the Anderson Family Strength and Conditioning Center. This team training requirement is in efforts to eliminate potential undue influence or pressure to participate in the research study. Total duration of exercise training each week will be approximately 8hrs. Below is the general weekly schedule of team training requirements and duration.

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday & Sunday
AM	Optional Weights .75 hours	Off	Optional Weights .75 hours	Off	Optional Weights .75 hours	Off
PM	Swim 2.0 hours	Swim 1.5 hours	Swim 1.5 hours	Swim 1.5 hours	Swim 1.5 hours	Off

In addition to the required six week intensified exercise training regime, if you participate in the research study you will be instructed throughout the study to orally ingest an encapsulated dosage of 1 Billion count of the probiotic B.infantis 35624 or 5g Maltodextrin daily as well as follow the week by week procedure requirements to complete the research study as described below.

Week 0 - Each subject will fill out a health history questionnaire to establish they qualify for the study, and will sign an informed consent statement. If agreeing to participate, all subjects will then complete a body composition measurement via GE Lunar iDXA technology, be provided supplementation instructions, receive sports nutrition education to ensure appropriate dietary intake, and given a three day dietary food log to complete.

Week 0 – Three days later that same week, 72 hrs before start of exercise training, all participants will return completed dietary food log to be randomized and complete all baseline testing which will include the following: a blood sample, RESTQ-Sport Psychology Questionnaire, force plate power test (Anderson Center), and Anaerobic (100m prime stroke time trial) and Aerobic (500 meter freestyle time trial) swim test.

Week 1 – All participants will begin the high fatiguing intensified training phase lead by head coach and team’s strength & conditioning coach, supplementation, and ending the training week by completing another RESTQ-Sport Psychology Questionnaire.

Week 2 – Begin week by completing a Week 1 Supplement Adherence Questionnaire to ensure supplement is being ingested according to study's protocol, continue high fatiguing intensified exercise training phase and supplementation throughout week followed by completing another RESTQ-Sport Psychology Questionnaire at conclusion of training week.

Week 3 - Begin week by completing a Week 2 Supplement Adherence Questionnaire, continue high fatiguing intensified exercise training phase and supplementation throughout week followed by mid-point testing which include at conclusion of training week collecting a RESTQ-Sport Psychology Questionnaire, three-day diet record, blood sample, force plate power test, and anaerobic & aerobic swim test. Week 4 - Begin week by completing a Week 3 Supplement Adherence Questionnaire, continue high fatiguing intensified exercise training phase and supplementation throughout week followed by completing another RESTQ-Sport Psychology Questionnaire at conclusion of training week.

Week 5 - Begin week by completing a Week 4 Supplement Adherence Questionnaire, continue high fatiguing intensified exercise training phase and supplementation throughout week followed by completing another RESTQ-Sport Psychology Questionnaire at conclusion of training week.

Week 6 - Begin week by completing a Week 5 Supplement Adherence Questionnaire, complete the high fatiguing intensified exercise training phase and supplementation followed by post-study testing which includes at conclusion of training week the collection of a RESTQ-Sport Psychology Questionnaire, three-day diet record, blood sample, force plate power test, anaerobic & aerobic swim test, and body composition measurement.

This study will abide stringently to HIPAA and your personal health information. All of your blood sample analysis results, informed consent, health history questionnaire, and RESTQ-Sport Psychological Questionnaire will be securely stored in the Applied Physiology Laboratory. All GE Lunar iDXA bone and body composition measurements will be saved electronically on a password protected DXA computer that operates the machine as well as the Kansas Athletics Sports Medicine Electronic Medical Records.

You are volunteering to participate in a study in which your body composition will be assessed using Dual-Energy X-Ray Absorptiometry, commonly referred to as DXA. This research study involves a procedure that uses x-rays. DXA is approved by the State of Kansas and the Radiation Safety Committee on campus for this specific use. The DXA measurements are being made by trained personnel who have received formal GE training following installation of the DXA for total body scans. Dr. Larry Magee, M.D., is a Board Certified advisor and medical consultant for the research study and will provide medical assistance and supervision for those who voluntarily participate in this study.

The amount of radiation that you will receive from a DXA whole body scan is fifty times less than a chest x-ray. The Radiation Safety Officer at the University of Kansas oversees this work and can provide you with more information about radiation exposure if you are interested.

RISKS

There is inherent danger in all physical exercise. You may experience muscle soreness during the six week research study. There is also the possibility of injury to your shoulder, knees, hips or back when performing the activities in this study. You may also experience some bruising or discomfort at the site of the blood sampling. You will be given a 24 hour contact number for study personnel to convey any type of unusual discomfort or injury. This study also involves exposure to x-ray radiation since the GE Lunar DXA is an imaging technique that uses two low-dose x-ray beams with different levels of energy to measure bone and body composition. The Lunar DXA's x-ray beams use a very small dose of radiation and have been stated by GE to be equivalent to the same radiation exposure during a Trans-Pacific flight. If any female subject is pregnant or believes to be pregnant she will not be permitted to undergo a scan.

BENEFITS

We cannot promise any benefits to you or others from taking part in this research.

PAYMENT TO PARTICIPANTS

There will be no compensation for participation in this study.

PARTICIPANT CONFIDENTIALITY

Your name will not be associated in any publication or presentation with the information collected about you or with the research findings from this study. Instead, the researcher(s) will use a study number or a pseudonym rather than your name. Your identifiable information will not be shared unless (a) it is required by law or university policy, or (b) you give written permission. Permission granted on this date to use and disclose your information remains in effect indefinitely. By signing this form you give permission for the use and disclosure of your information for purposes of this study at any time in the future. All data from this study will be destroyed after it has been presented at a scientific conference and/or after it has been published in a scientific journal, and after a 7 year period.

INSTITUTIONAL DISCLAIMER STATEMENT

In the event of injury, the Kansas Tort Claims Act provides for compensation if it can be demonstrated that the injury was caused by the negligent or wrongful act or omission of a state employee acting within the scope of his/her employment.

REFUSAL TO SIGN CONSENT AND AUTHORIZATION

You are not required to sign this Consent and Authorization form and you may refuse to do so without affecting your right to any services you are receiving or may receive from the University of Kansas or to participate in any programs or events of the University of Kansas. Furthermore, participation or refusal to participate will not affect your relationship with Kansas Athletics, respective scholarship agreement, standing with the swim team, or academic standing. However, if you refuse to sign, you cannot participate in this study.

CANCELLING THIS CONSENT AND AUTHORIZATION

You may withdraw your consent to participate in this study at any time. You also have the right to cancel your permission to use and disclose further information collected about you, in writing, at any time, by sending your written request to: Dr. Andrew Fry, 1301 Sunnyside Ave., Room 101C.

If you cancel permission to use your information, the researchers will stop collecting additional information about you. However, the research team may use and disclose information that was gathered before they received your cancellation, as described above.

QUESTIONS ABOUT PARTICIPATION

Questions about procedures should be directed to the researcher(s) listed at the end of this consent form.

PARTICIPANT CERTIFICATION:

I have read this Consent and Authorization form. I have had the opportunity to ask, and I have received answers to, any questions I had regarding the study. I understand that if I have any additional questions about my rights as a research participant, I may call (785) 864-7429 or (785) 864-7385, write the Human Subjects Committee Lawrence Campus (HSCL), University of Kansas, 2385 Irving Hill Road, Lawrence, Kansas 66045-7568, or email irb@ku.edu.

I agree to take part in this study as a research participant. By my signature I affirm that I am at least 18 years old and that I have received a copy of this Consent and Authorization form.

Type/Print Participant's Name

Date

Participant's Signature

Researcher Contact Information

Aaron Carbuhn
Student Principal Investigator
Kansas Athletics, Inc.
1651 Naismith Dr.
University of Kansas
Lawrence, KS 66045
785 864 - 1846

Dr. Andrew Fry, PhD
Faculty Advisor Principal Investigator
HSES Dept., 101A Robinson
University of Kansas
Lawrence, KS 66045
785 864 - 4656

Appendix C
Pre-Participation Questionnaire

**PRE-EXERCISE TESTING
HEALTH & EXERCISE STATUS
QUESTIONNAIRE**



Name _____ Date _____

Home Address _____

Phone Number _____ Email _____

Birthday (mm/dd/yy) ____/____/____

Person to contact in case of emergency _____

Emergency Contact Phone _____

Gender _____ Age _____(yrs) Height _____(ft)_____(in) Weight _____(lbs)

A. JOINT-MUSCLE STATUS (✓Check areas where you currently have problems)

Joint Areas

- () Wrists
- () Elbows
- () Shoulders
- () Upper Spine & Neck
- () Lower Spine
- () Hips
- () Knees
- () Ankles
- () Feet
- () Other _____

Muscle Areas

- () Arms
- () Shoulders
- () Chest
- () Upper Back & Neck
- () Abdominal Regions
- () Lower Back
- () Buttocks
- () Thighs
- () Lower Leg
- () Feet
- () Other _____

B. HEALTH STATUS (✓Check if you currently have any of the following conditions)

- | | |
|---|--|
| <input type="checkbox"/> High Blood Pressure | <input type="checkbox"/> Acute Infection |
| <input type="checkbox"/> Heart Disease or Dysfunction | <input type="checkbox"/> Diabetes or Blood Sugar Level Abnormality |
| <input type="checkbox"/> Peripheral Circulatory Disorder | <input type="checkbox"/> Anemia |
| <input type="checkbox"/> Lung Disease or Dysfunction | <input type="checkbox"/> Hernias |
| <input type="checkbox"/> Arthritis or Gout | <input type="checkbox"/> Thyroid Dysfunction |
| <input type="checkbox"/> Edema | <input type="checkbox"/> Pancreas Dysfunction |
| <input type="checkbox"/> Epilepsy | <input type="checkbox"/> Liver Dysfunction |
| <input type="checkbox"/> Multiple Sclerosis | <input type="checkbox"/> Kidney Dysfunction |
| <input type="checkbox"/> High Blood Cholesterol or
Triglyceride Levels | <input type="checkbox"/> Phenylketonuria (PKU) |
| <input type="checkbox"/> Allergic reactions to rubbing alcohol | <input type="checkbox"/> Loss of Consciousness |

* NOTE: If any of these conditions are checked, then a physician's health clearance will be required.

C. PHYSICAL EXAMINATION HISTORY

Approximate date of your last physical examination _____

Physical problems noted at that time _____

Has a physician ever made any recommendations relative to limiting your level of physical exertion? _____ YES _____ NO

If YES, what limitations were recommended? _____

D. CURRENT MEDICATION USAGE (List the drug name, the condition being managed, and the length of time used)

MEDICATION

CONDITION

LENGTH OF USAGE

E. PHYSICAL PERCEPTIONS (Indicate any unusual sensations or perceptions. ✓Check if you have recently experienced any of the following during or soon after *physical activity* (PA); or during *sedentary periods* (SED))

<u>PA</u>	<u>SED</u>		<u>PA</u>	<u>SED</u>	
<input type="checkbox"/>	<input type="checkbox"/>	Chest Pain	<input type="checkbox"/>	<input type="checkbox"/>	Nausea
<input type="checkbox"/>	<input type="checkbox"/>	Heart Palpitations	<input type="checkbox"/>	<input type="checkbox"/>	Light Headedness
<input type="checkbox"/>	<input type="checkbox"/>	Unusually Rapid Breathing	<input type="checkbox"/>	<input type="checkbox"/>	Loss of Consciousness
<input type="checkbox"/>	<input type="checkbox"/>	Overheating	<input type="checkbox"/>	<input type="checkbox"/>	Loss of Balance
<input type="checkbox"/>	<input type="checkbox"/>	Muscle Cramping	<input type="checkbox"/>	<input type="checkbox"/>	Loss of Coordination
<input type="checkbox"/>	<input type="checkbox"/>	Muscle Pain	<input type="checkbox"/>	<input type="checkbox"/>	Extreme Weakness
<input type="checkbox"/>	<input type="checkbox"/>	Joint Pain	<input type="checkbox"/>	<input type="checkbox"/>	Numbness
<input type="checkbox"/>	<input type="checkbox"/>	Other_____	<input type="checkbox"/>	<input type="checkbox"/>	Mental Confusion

F. FAMILY HISTORY (✓Check if any of your blood relatives . . . parents, brothers, sisters, aunts, uncles, and/or grandparents . . . have or had any of the following)

- ☐ Heart Disease
- ☐ Heart Attacks or Strokes (prior to age 50)
- ☐ Elevated Blood Cholesterol or Triglyceride Levels
- ☐ High Blood Pressure
- ☐ Diabetes
- ☐ Sudden Death (other than accidental)

G. STUDY EXCLUSION CRITERIA (✓Check if applicable)

- ☐ Pregnant
- ☐ Diagnosed Lactose Intolerance
- ☐ Diagnosed Immunodeficiency
- ☐ Any Previous Abdominal Surgery (with exception to hernia repair or appendectomy)
- ☐ Documented Psychiatric Illness
- ☐ Have taken any anti-inflammatory medications or antibiotics for the previous month

* NOTE: If any of these conditions are checked, you will be excluded from the study.

Appendix D
RESTQ52-Sport Questionnaire

APPENDIX D: RECOVERY STRESS QUESTIONNAIRE (RESTQ-52 Sport)

This questionnaire consists of a series of statements. These statements possibly describe your psychic or physical wellbeing or your activities during the past few days and nights. Please select the answer that most accurately reflects your thoughts and activities. Indicate how often each statement was right in your case in the past few days. The statements related to performance should refer to performance during competition as well as during practice.

For each statement there are seven possible answers.

Please make your selection by marking the number corresponding to the appropriate answer. Example:

In the past (3) days/nights

... I read a newspaper

0	1	2	3		X	
				4	5	6
never	seldom	sometime	often	more often	very often	always

In this example, the number 5 is marked. This means that you read a newspaper very often in the past three days.

Please do not leave any statements blank.

If you are unsure which answer to choose, select the one that most closely applies to you. Please turn the page and respond to the statements in order without interruption.

In the past 3 days/nights

1)... 1 watched TV

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

2)... I laughed

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

3)... 1 was in a bad mood

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

4)... I felt physically relaxed

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

5)... 1 was in good spirits

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

6)... I had difficulties in concentrating

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

7)... I worried about unresolved problems

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

8)... 1 had a good time with my friends

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

9)... 1 had a headache

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

10)... I was dead tired after work

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

11)... I was successful in what I did

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

12)... I felt uncomfortable

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

13)... I was annoyed by others

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

14)... I felt down

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

15)... I had a satisfying sleep

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

16)... I was fed up with everything

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

17)... I was in a good mood

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

18)... I was overtired

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

19)... I slept restlessly

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

20)... I was annoyed

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

21)... I felt as though I could get everything done

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

22)... I was upset

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

23)... I put off making decisions

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

24)... I made important decisions

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

25)... I felt under pressure

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

26)... parts of my body were aching

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

27)...I could not get rest during the breaks

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

28)... I was convinced I could achieve my set goals during performance

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

29)... I recovered well physically

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

30)... I felt burned out by my sport

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

31)... I accomplished many worthwhile things in my sport

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

32)... I prepared myself mentally for performance

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

33)... my muscles felt stiff or tense during performance

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

34)... 1 had the impression there were too few breaks

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

35)... 1 was convinced that I could achieve my performance at any time

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

36)... I dealt effectively with my team-mates' problems

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

37)... I was in good condition physically

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

38)... I pushed myself during performance

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

39)... I felt emotionally drained from performance

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

40)... 1 had muscle pain after performance

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

41)... I was convinced that I performed well

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

42)... too much was demanded of me during breaks

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

43)... 1 psyched myself up before performance

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

44)... I felt I wanted to quit my sport

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

45)... I felt very energetic

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

46)... I easily understand how my team mates felt about things

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

47)... I was convinced that I had trained well

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

48)... the breaks were not right at times

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

49)... I felt vulnerable to injuries

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

50)... I set definite goals for myself during performance

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

51)... my body felt strong

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

52)... I felt frustrated by my sport

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

53)... I dealt with emotional problems in my sport very calmly

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very	always

Thank you very much

Appendix E
Dietary Food Log

Food Diary

Instructions

To assist you in reaching your goals for fueling athletic performance and to customize a nutrition plan for you, it is important to know your current eating habits. On the following pages, please write down everything you eat and drink for 3 days. Try to pick 3 days that are "typical" of the way you eat. If possible, choose 2 weekdays and 1 weekend day. Do not try to change your eating habits during the 3 days of record keeping.

If you have any questions about completing this form, please contact your sports dietitian at:

Helpful Hints

Record what you have eaten as soon as possible after meals. This makes it much easier to remember what and how much you eat. Remember the following:

- **Preparation:** How was the food cooked? Was it baked, grilled, fried, steamed, or baked? Was it fresh, frozen or canned?
 - **Portion size:** Indicate how much of each food you eat by using cups, ounces, teaspoons, or tablespoons, or a "handful" where possible. For meats, estimate the ounces you eat. (A deck of cards or a computer mouse is about a 3-ounce portion.)
 - **Include the fluids that you drink.** List the amounts and the types, and the times that you drink them.
 - **Include the "extras" or condiments you eat:** Do you put cream or sugar in coffee? Is your tea sweetened or unsweetened? Do you use ketchup, mustard, mayonnaise, steak sauce, or salsa on foods?
 - **Be specific:** If you eat bread, is it white, wheat, whole wheat, rye, honey wheat or multigrain? If you drink milk, it is whole, 2%, 1%, skim, soy, or rice milk?
-

Appendix F

All Dependent Variable Figures, Tables

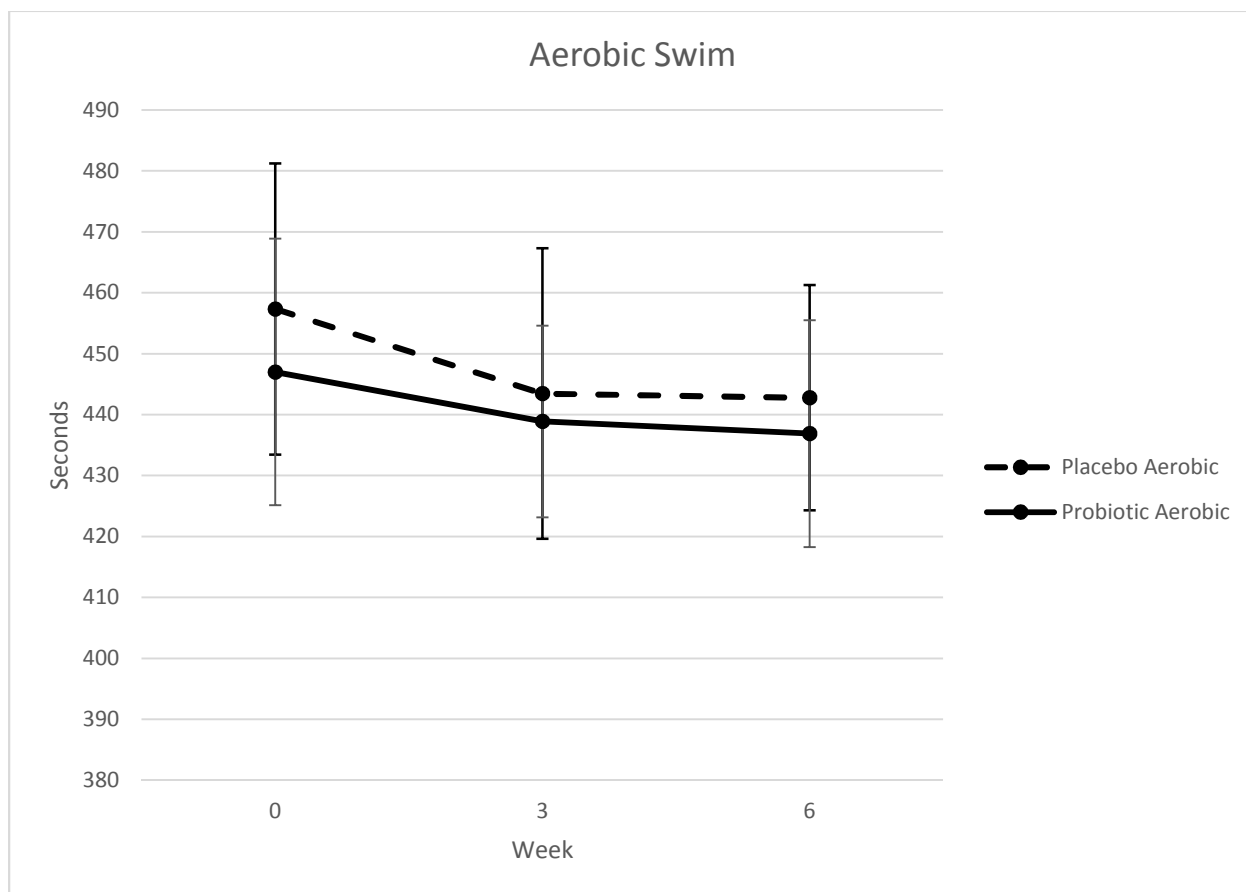
Figure 1 – 100m Aerobic Freestyle Swim Time Trial

Figure 1 100m aerobic freestyle swim time trial (seconds) in collegiate female swimmers pre, mid, and post 6 weeks of exercise training and treatment. Probiotic the *B. infantis*35624 supplemented group, Placebo group, PRE week 0, MID week 3, POST week 6; n = 8 (*B. infantis*35624 supplementation), n = 9 (placebo). Values are means \pm SD.

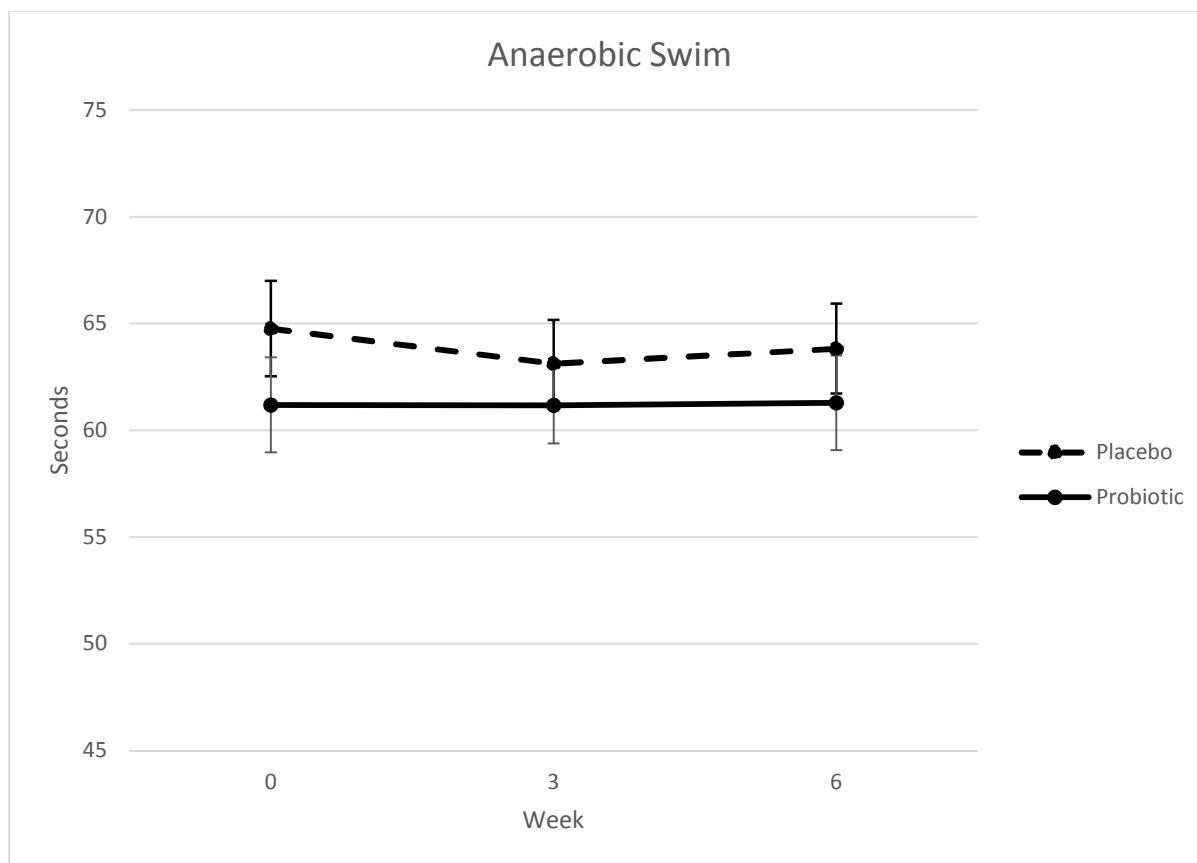
Figure 2 – 500m Anaerobic Freestyle Swim Time Trial

Figure 2 500m anaerobic freestyle swim time trial (seconds) in collegiate female swimmers pre, mid, and post 6 weeks of exercise training and treatment. *Probiotic* the *B. infantis*35624 supplemented group, *Placebo* group, *PRE* week 0, *MID* week 3, *POST* week 6; n = 8 (*B. infantis*35624 supplementation), n = 9 (placebo). Values are means \pm SD.

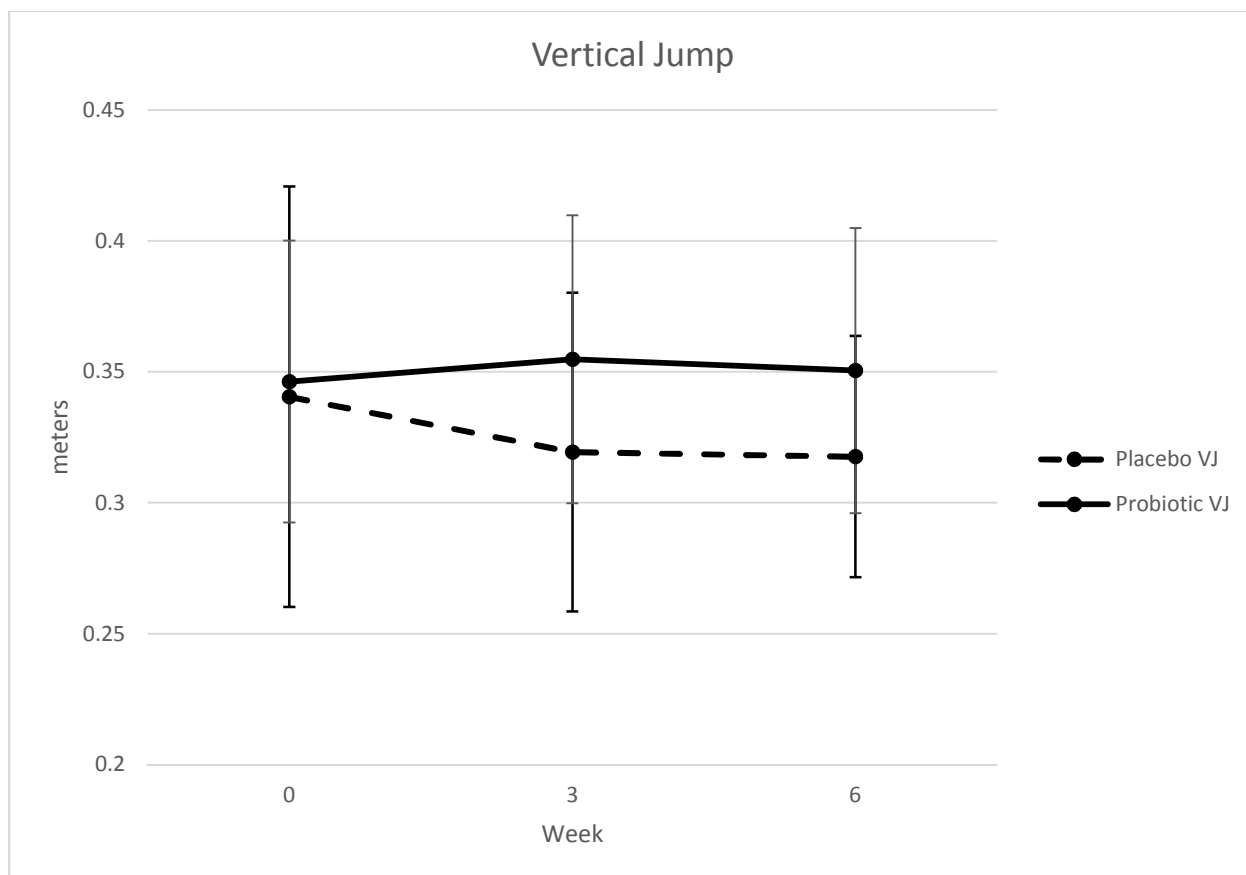
Figure 3 – Force Plate Vertical Jump

Figure 3 Force plate vertical jump (meters) in collegiate female swimmers pre, mid, and post 6 weeks of exercise training and treatment. *Probiotic* the *B. infantis*35624 supplemented group, *Placebo* group, *PRE* week 0, *MID* week 3, *POST* week 6; n = 8 (*B. infantis*35624 supplementation), n = 9 (placebo). Values are means \pm SD.

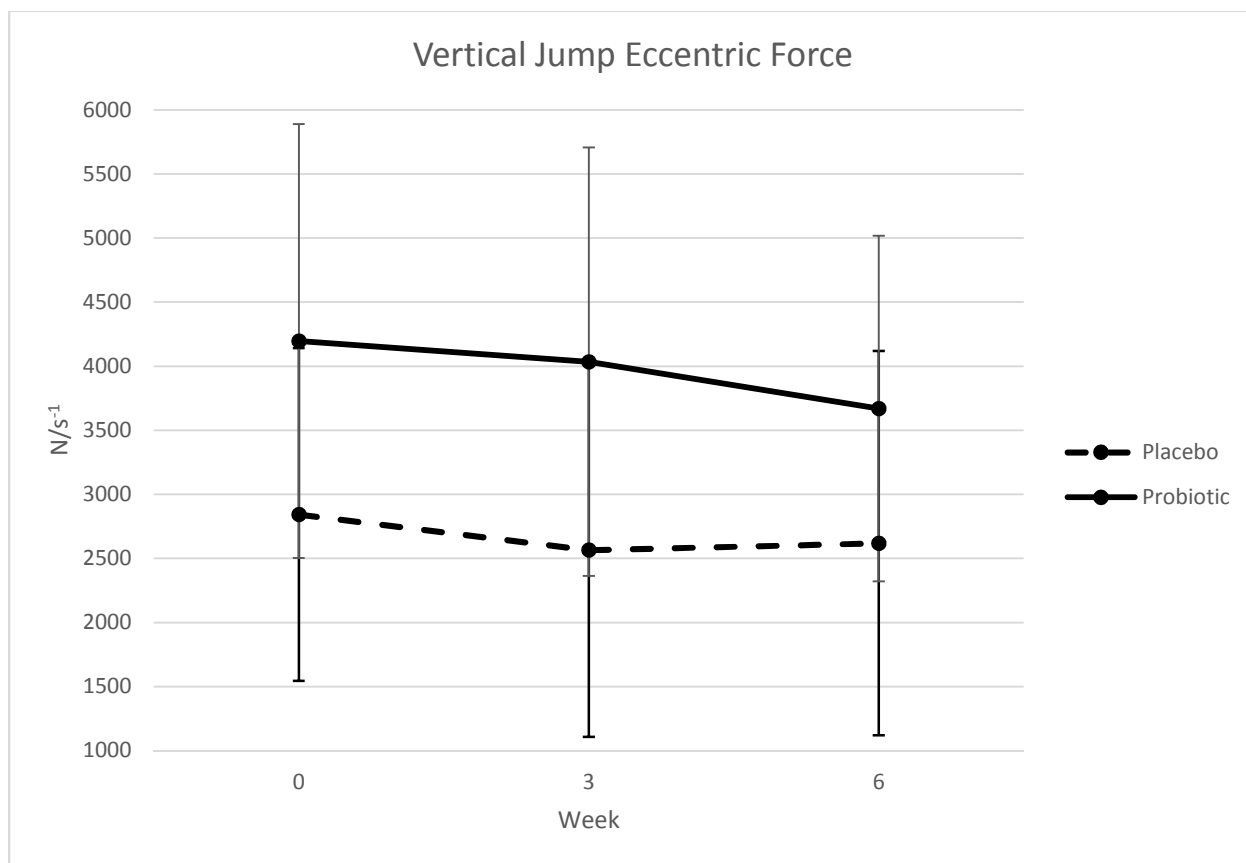
Figure 4 – Force Plate Vertical Jump Eccentric Force

Figure 4 Force plate vertical jump eccentric force (N/s⁻¹) in collegiate female swimmers pre, mid, and post 6 weeks of exercise training and treatment. Probiotic the B. infantis35624 supplemented group, Placebo group, PRE week 0, MID week 3, POST week 6; n = 8 (B. infantis35624 supplementation), n = 9 (placebo). Values are means \pm SD.

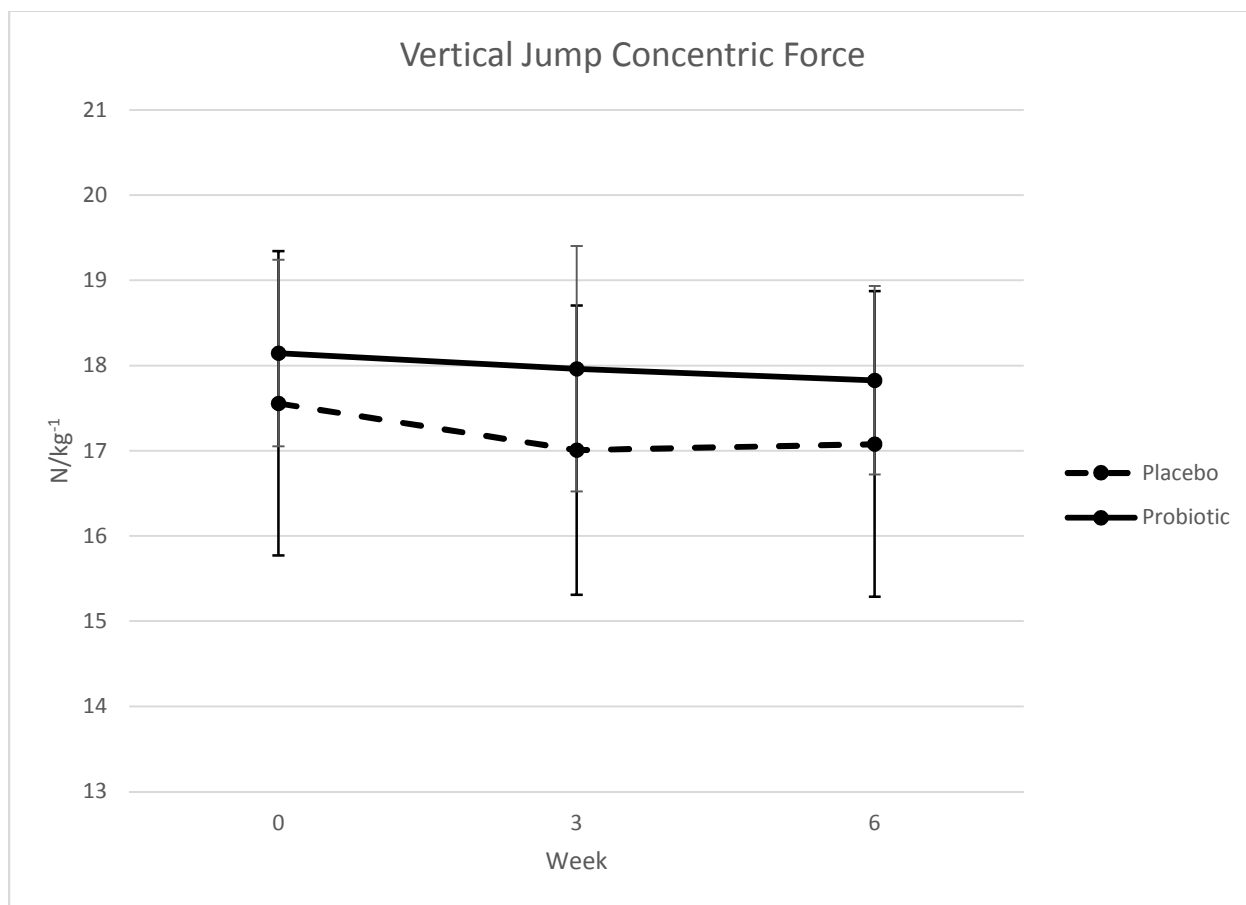
Figure 5 – Force Plate Vertical Jump Concentric Force

Figure 5 Force plate vertical jump concentric force (N/kg⁻¹) in collegiate female swimmers pre, mid, and post 6 weeks of exercise training and treatment. *Probiotic* the *B. infantis*35624 supplemented group, *Placebo* group, *PRE* week 0, *MID* week 3, *POST* week 6; n = 8 (*B. infantis*35624 supplementation), n = 9 (placebo). Values are means \pm SD.

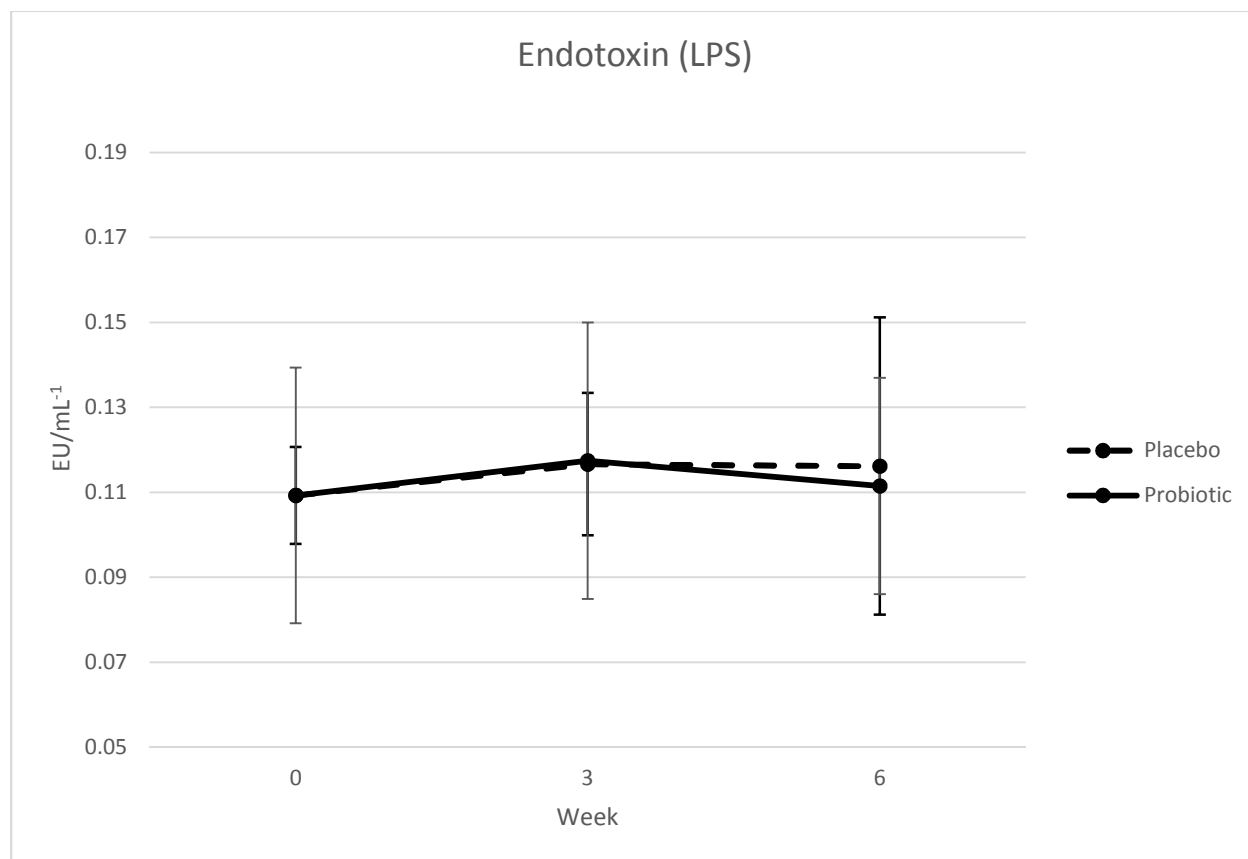
Figure 6 – Serum Endotoxin (LPS)

Figure 6 Serum concentrations of endotoxin (LPS) in collegiate female swimmers pre, mid, and post 6 weeks of exercise training and treatment. *Probiotic* the *B. infantis*35624 supplemented group, *Placebo* group, *PRE* week 0, *MID* week 3, *POST* week 6; n = 6 (*B. infantis*35624 supplementation), n = 7 (placebo). Values are means \pm SD.

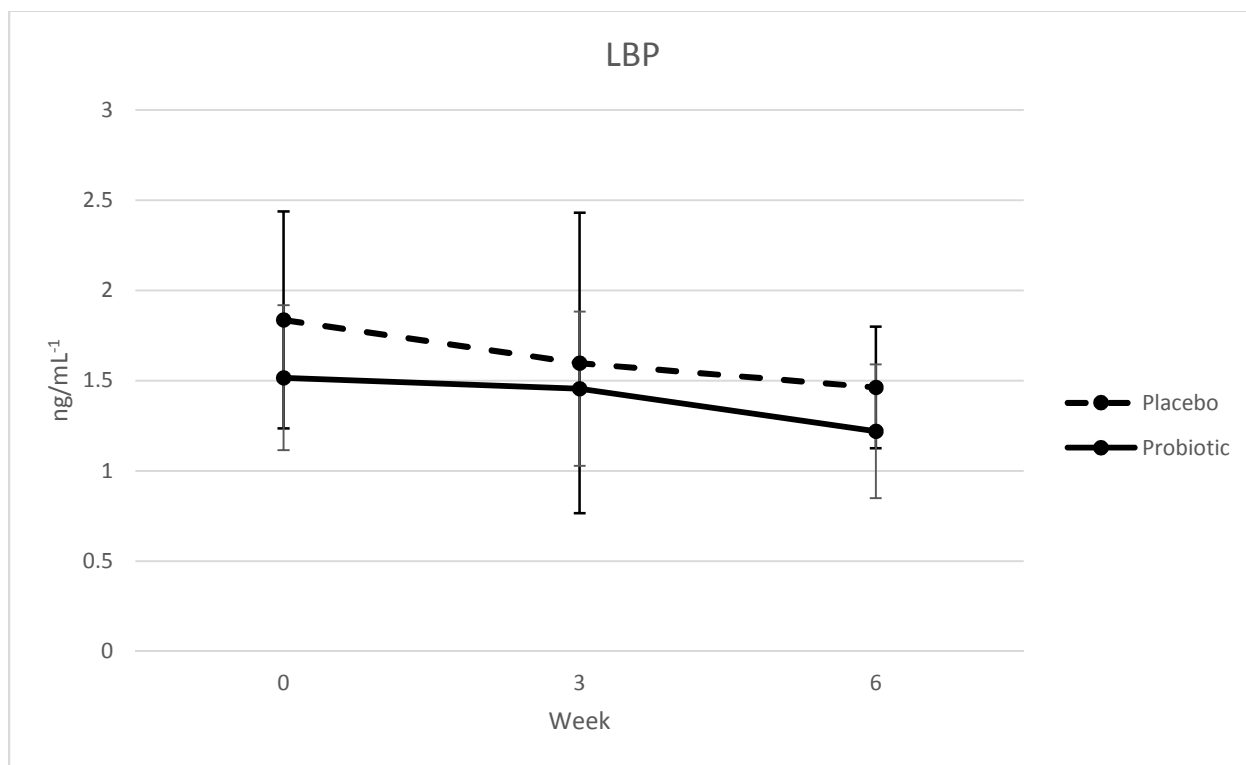
Figure 7 – Serum LBP

Figure 7 Serum concentrations of LBP (ng/mL⁻¹) in collegiate female swimmers pre, mid, and post 6 weeks of exercise training and treatment. Probiotic the B. infantis35624 supplemented group, Placebo group, PRE week 0, MID week 3, POST week 6; n = 8 (B. infantis35624 supplementation), n = 9 (placebo). Values are means \pm SD.

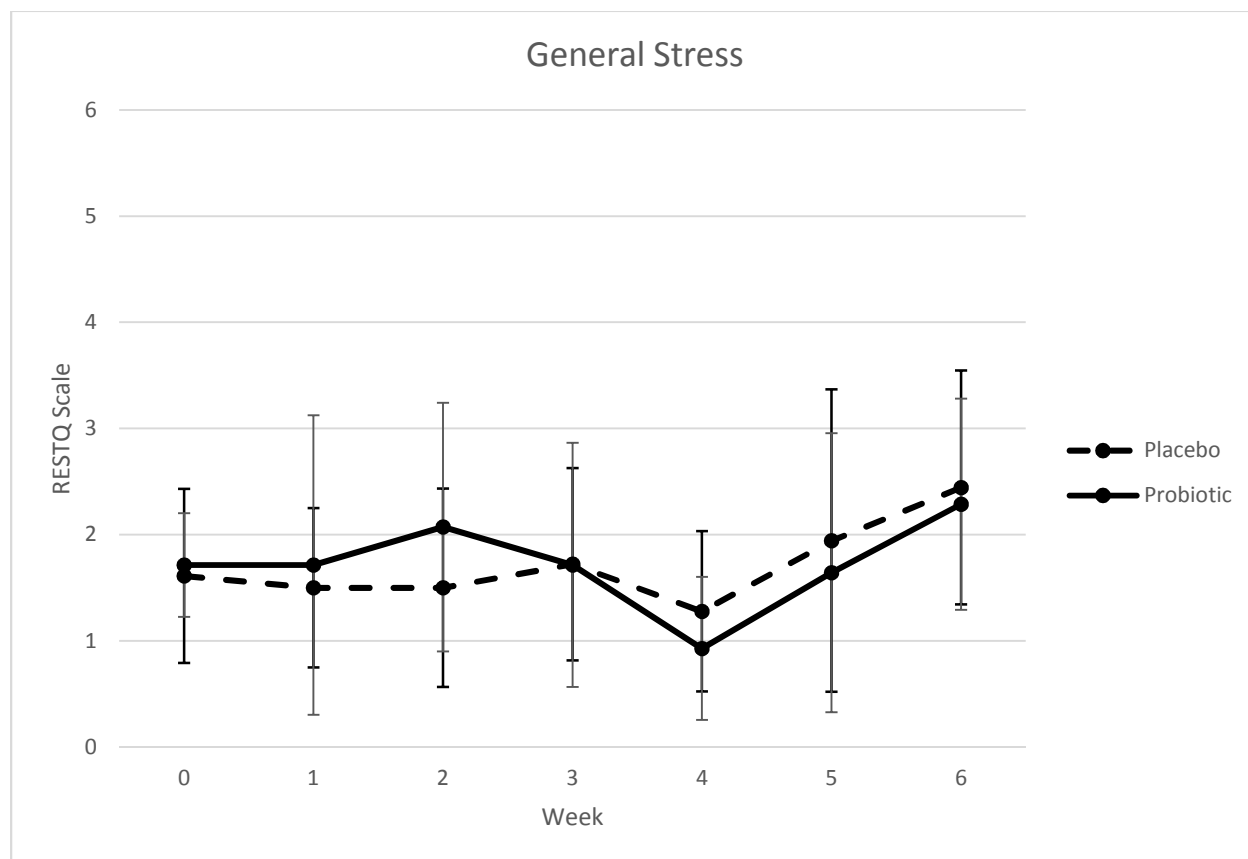
Figure 8 – RESTQ52-Sport Scale – General Stress

Figure 8 RESTQ52-Sport weekly general stress scores in collegiate female swimmers in 6 weeks of exercise training and treatment. Subjects with high values describe themselves as being frequently mentally stressed, depressed, unbalanced, and listless. RESTQ52-Sport scale is defined as 0-never, 1-seldom, 2-sometimes, 3-often, 4-more often, 5-very often, 6-always. *Probiotic* the B. infantis35624 supplemented group, *Placebo* group, Week 0-6; n = 7 (B. infantis35624 supplementation), n = 9 (placebo). Values are means \pm SD.

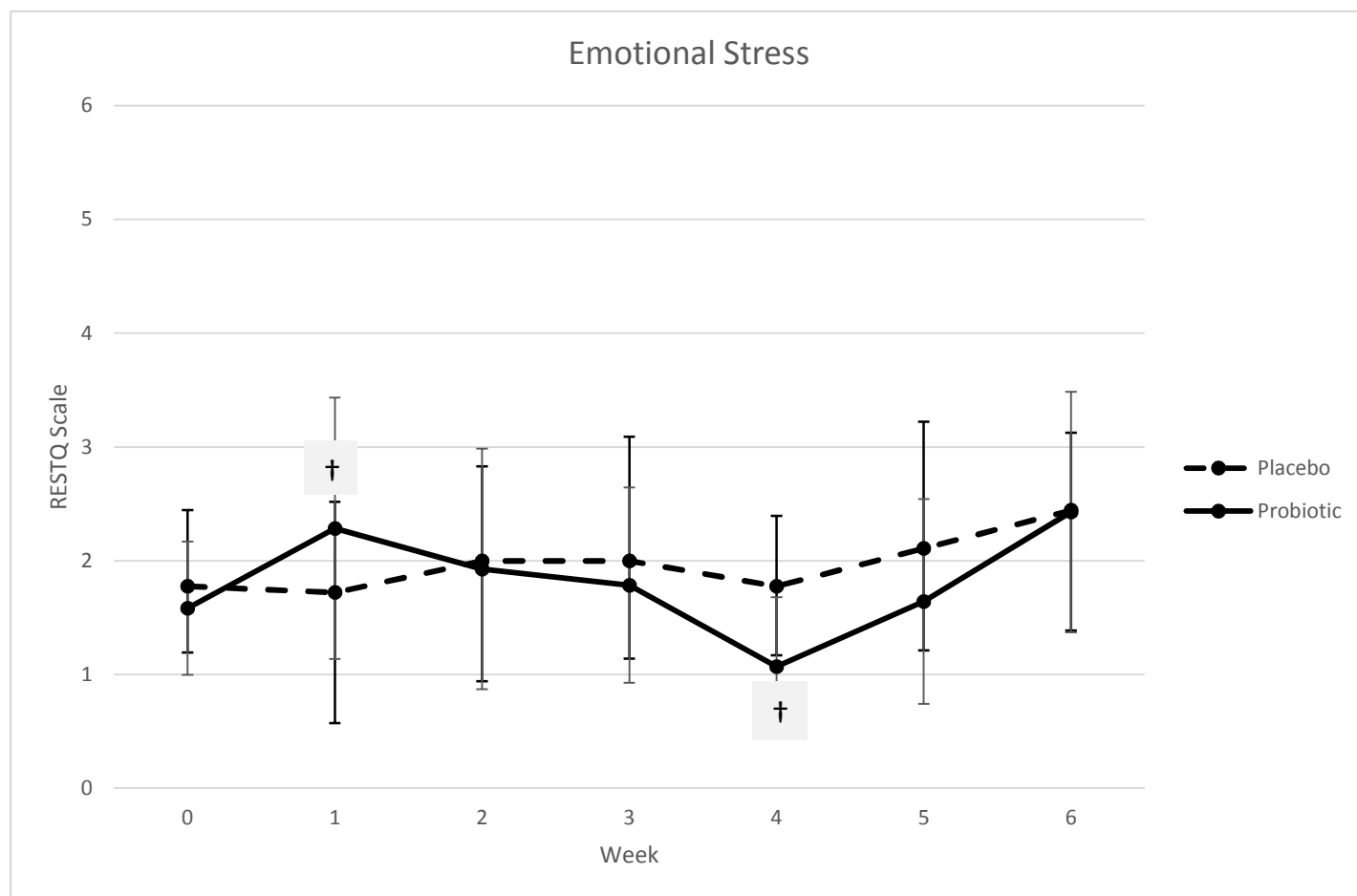
Figure 9 – RESTQ52-Sport Scale – Emotional Stress

Figure 9 RESTQ52-Sport weekly emotional stress scores in collegiate female swimmers in 6 weeks of exercise training and treatment. Subjects with high values experience frequent irritation, aggression, anxiety, and inhibition. RESTQ52-Sport scale is defined as 0-never, 1-seldom, 2-sometimes, 3-often, 4-more often, 5-very often, 6-always. *Probiotic* the B. infantis35624 supplemented group, *Placebo* group, Week 0-6; n = 7 (B. infantis35624 supplementation), n = 9 (placebo). Values are means \pm SD. There was a trend towards significant difference between groups at week 1 and 4 of exercise training and treatment: † P < 0.10 (ANCOVA).

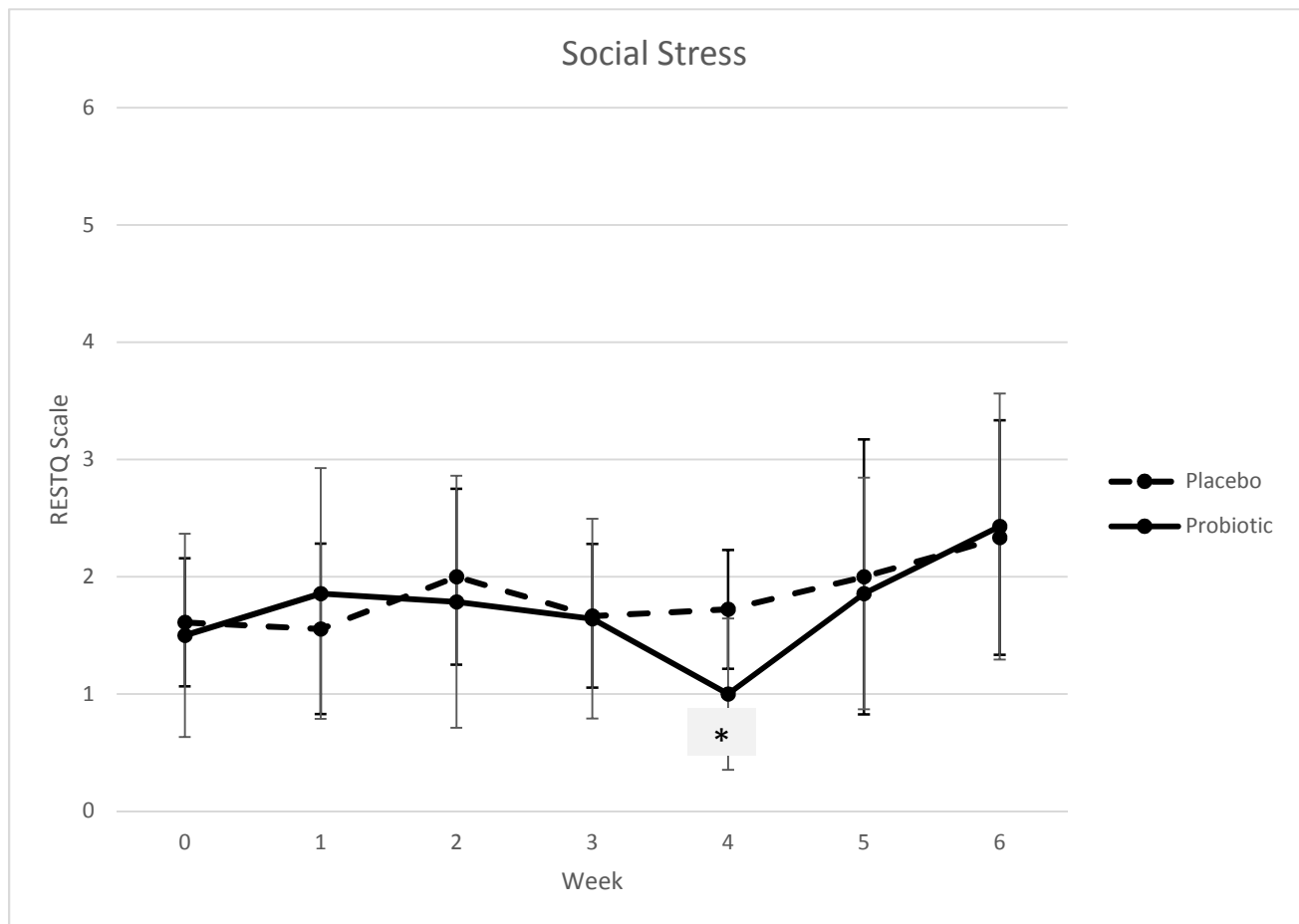
Figure 10 – RESTQ52-Sport Scale – Social Stress

Figure 10 RESTQ52-Sport weekly social stress scores in collegiate female swimmers in 6 weeks of exercise training and treatment. High values match subjects with frequent arguments, fights, irritation concerning others, general upset, and lack of humor. RESTQ52-Sport scale is defined as 0-never, 1-seldom, 2-sometimes, 3-often, 4-more often, 5-very often, 6-always. *Probiotic* the B. infantis35624 supplemented group, *Placebo* group, Week 0-6; n = 7 (B. infantis35624 supplementation), n = 9 (placebo). Values are means ± SD. There was a significant difference between groups at week 4 of exercise training and treatment: * P < 0.05 (ANCOVA).

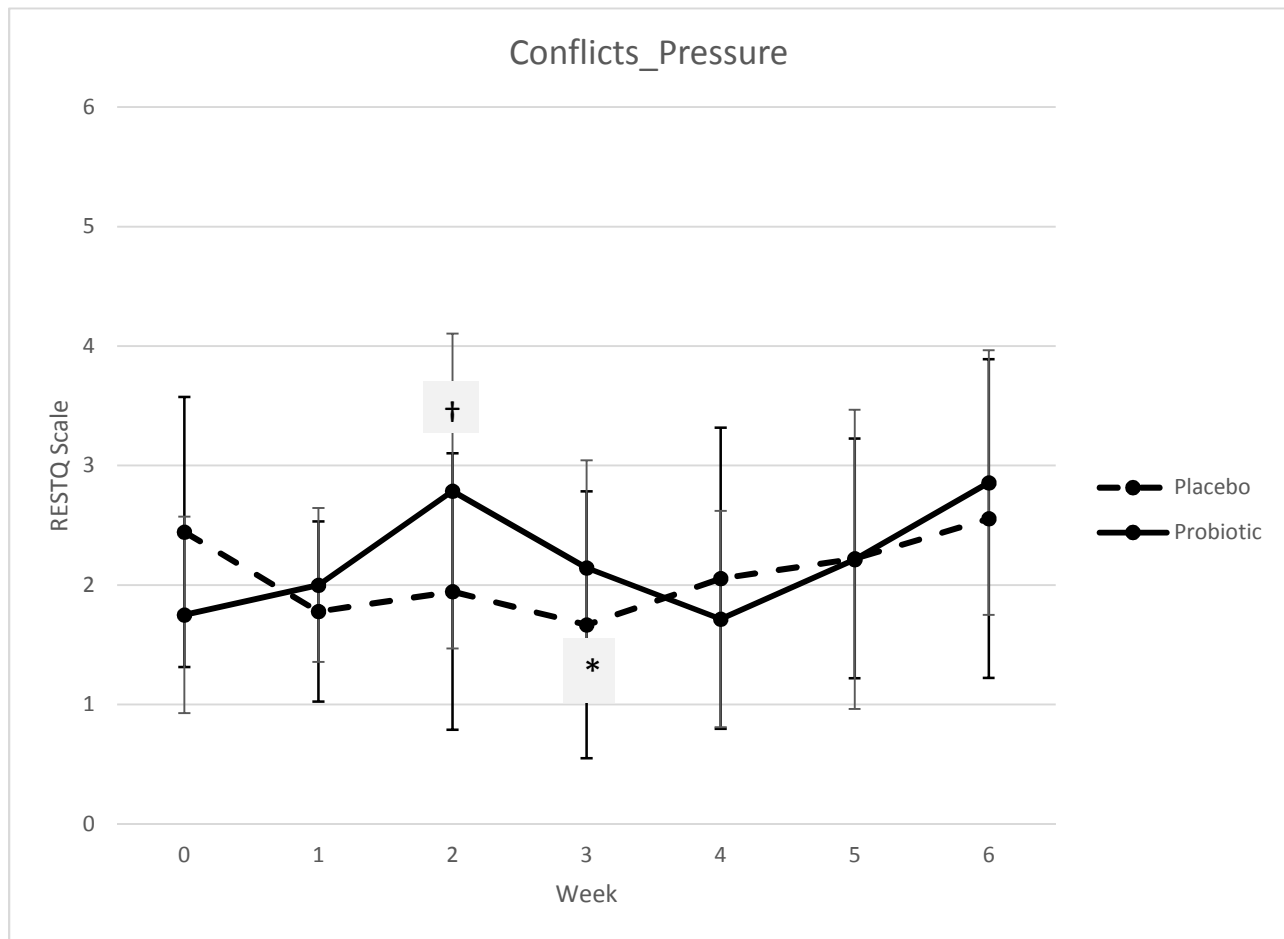
Figure 11 – RESTQ52-Sport Scale – Conflicts_Pressure

Figure 11 RESTQ52-Sport weekly conflicts_pressure scores in collegiate female swimmers in 6 weeks of exercise training and treatment. High values are reached if in the preceding few days conflicts were unsettled, unpleasant things had to be done, goals could not be reached, and certain thoughts could not be dismissed. RESTQ52-Sport scale is defined as 0-never, 1-seldom, 2-sometimes, 3-often, 4-more often, 5-very often, 6-always. *Probiotic* the *B. infantis*35624 supplemented group, *Placebo* group, Week 0-6; n = 7 (*B. infantis*35624 supplementation), n = 9 (placebo). Values are means \pm SD. There was a trend towards significant difference between groups at week 2 ($\dagger P < 0.10$) and a significant difference at week 3 of exercise training and treatment: $* P \leq 0.05$ (ANCOVA).

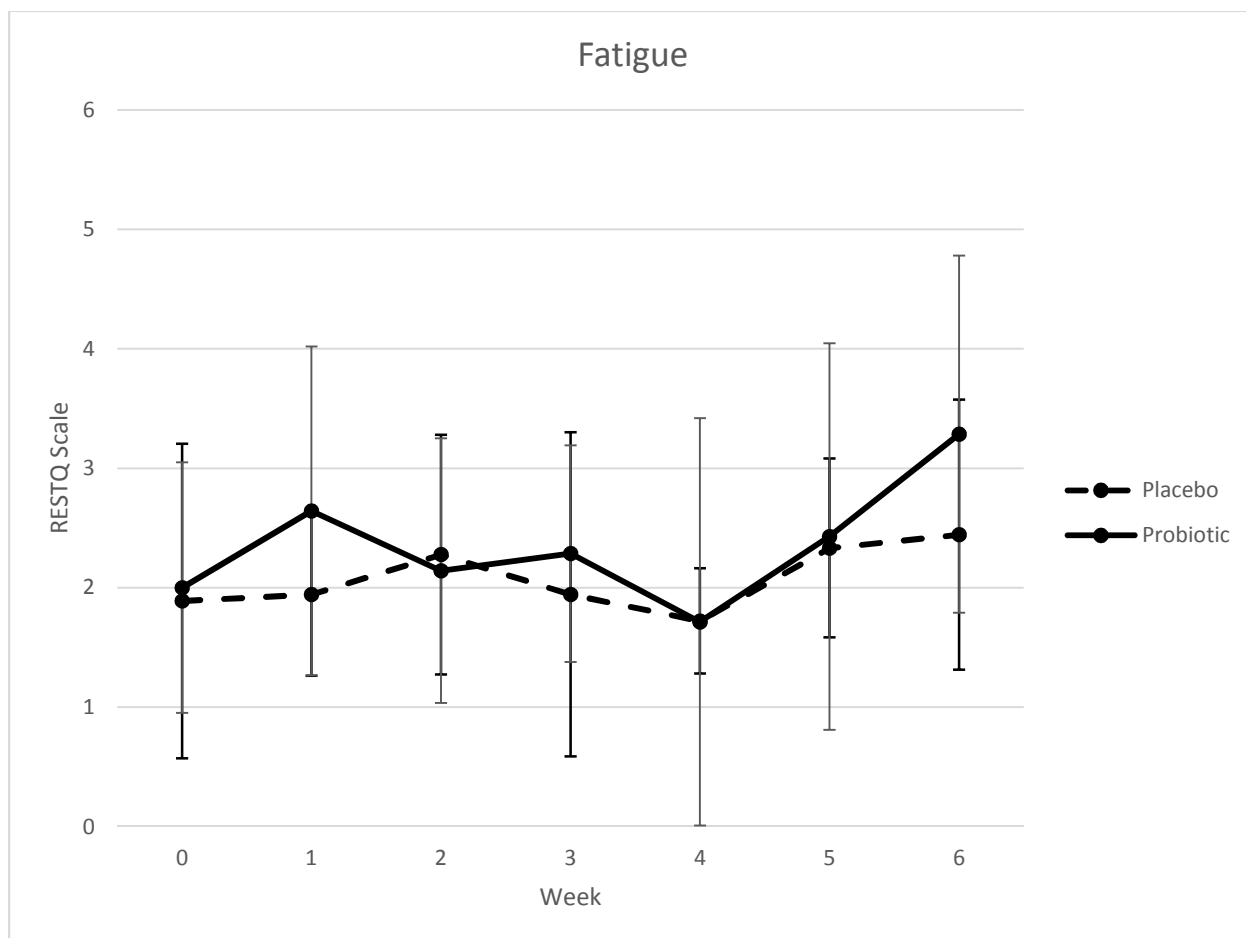
Figure 12 – RESTQ52-Sport Scale – Fatigue

Figure 12 RESTQ52-Sport weekly fatigue scores in collegiate female swimmers in 6 weeks of exercise training and treatment. Time pressure in job, training, school, and life, being constantly disturbed during important work, over-fatigue, and lack of sleep characterize this area of stress. RESTQ52-Sport scale is defined as 0-never, 1-seldom, 2-sometimes, 3-often, 4-more often, 5-very often, 6-always. *Probiotic* the *B. infantis*35624 supplemented group, *Placebo* group, *Week* 0-6; n = 7 (*B. infantis*35624 supplementation), n = 9 (placebo). Values are means \pm SD.

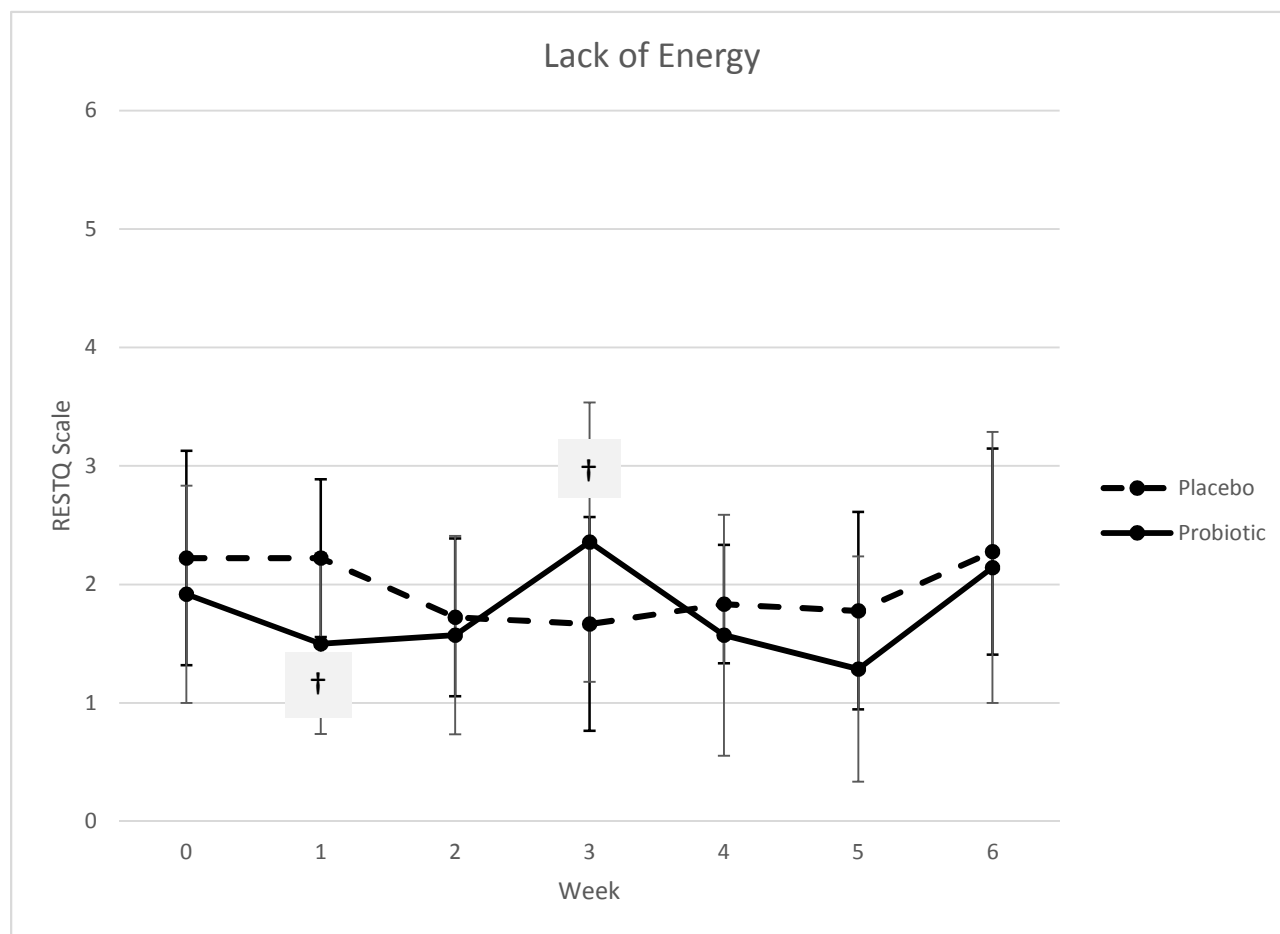
Figure 13 – RESTQ52-Sport Scale – Lack of Energy

Figure 13 RESTQ52-Sport weekly lack of energy scores in collegiate female swimmers in 6 weeks of exercise training and treatment. This score match's ineffective work behavior like inability to concentrate and lack of energy and decision making. RESTQ52-Sport scale is defined as 0-never, 1-seldom, 2-sometimes, 3-often, 4-more often, 5-very often, 6-always. *Probiotic* the B. infantis35624 supplemented group, *Placebo* group, *Week* 0-6; n = 7 (B. infantis35624 supplementation), n = 9 (placebo). Values are means ± SD. There was a trend towards significant difference between groups at week 1 and 3 of exercise training and treatment: † P < 0.10 (ANCOVA).

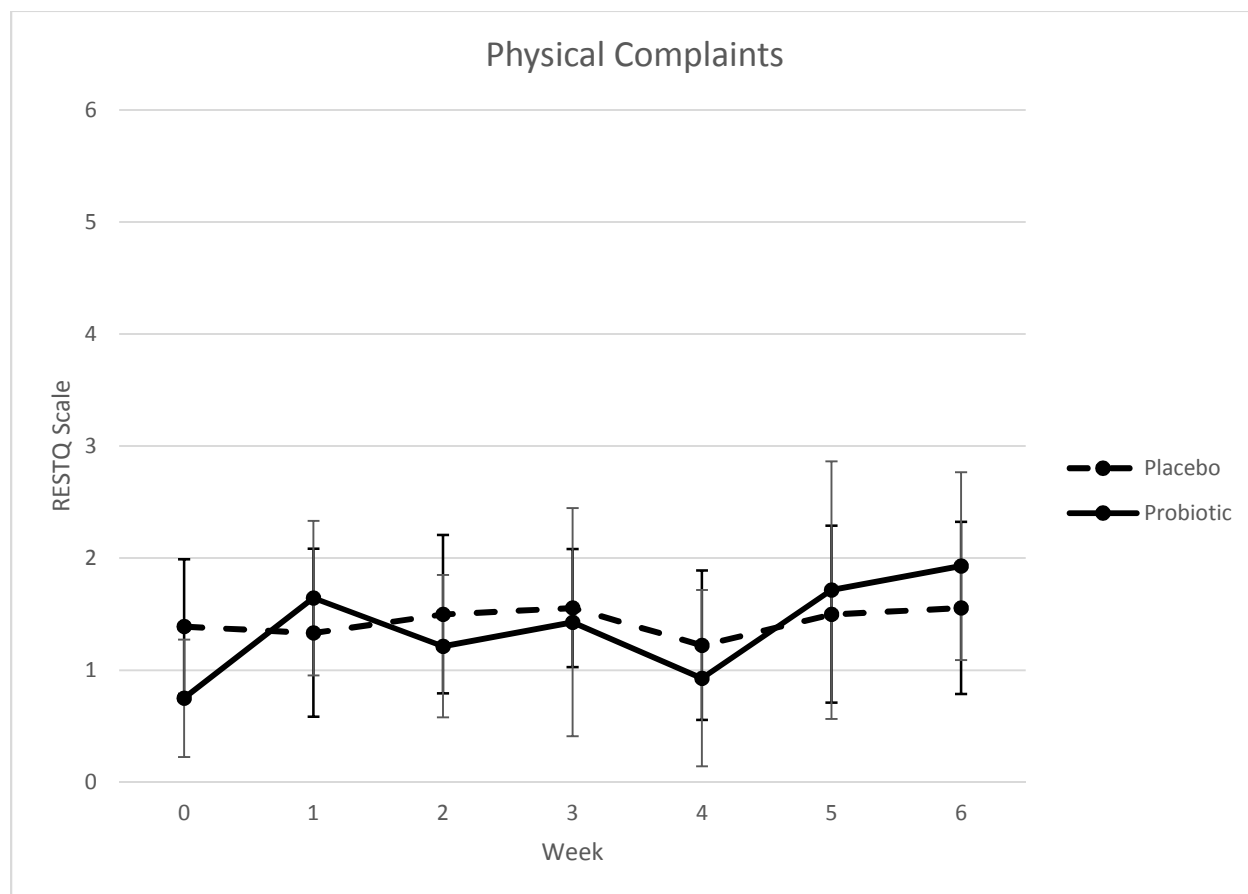
Figure 14 – RESTQ52-Sport Scale – Physical Complaints

Figure 14 RESTQ52-Sport weekly physical complaints scores in collegiate female swimmers in 6 weeks of exercise training and treatment. Physical indisposition and physical complaints related to the whole body are characterized by this scale. RESTQ52-Sport scale is defined as 0-never, 1-seldom, 2-sometimes, 3-often, 4-more often, 5-very often, 6-always. *Probiotic* the B. infantis35624 supplemented group, *Placebo* group, *Week* 0-6; n = 7 (B. infantis35624 supplementation), n = 9 (placebo). Values are means \pm SD.

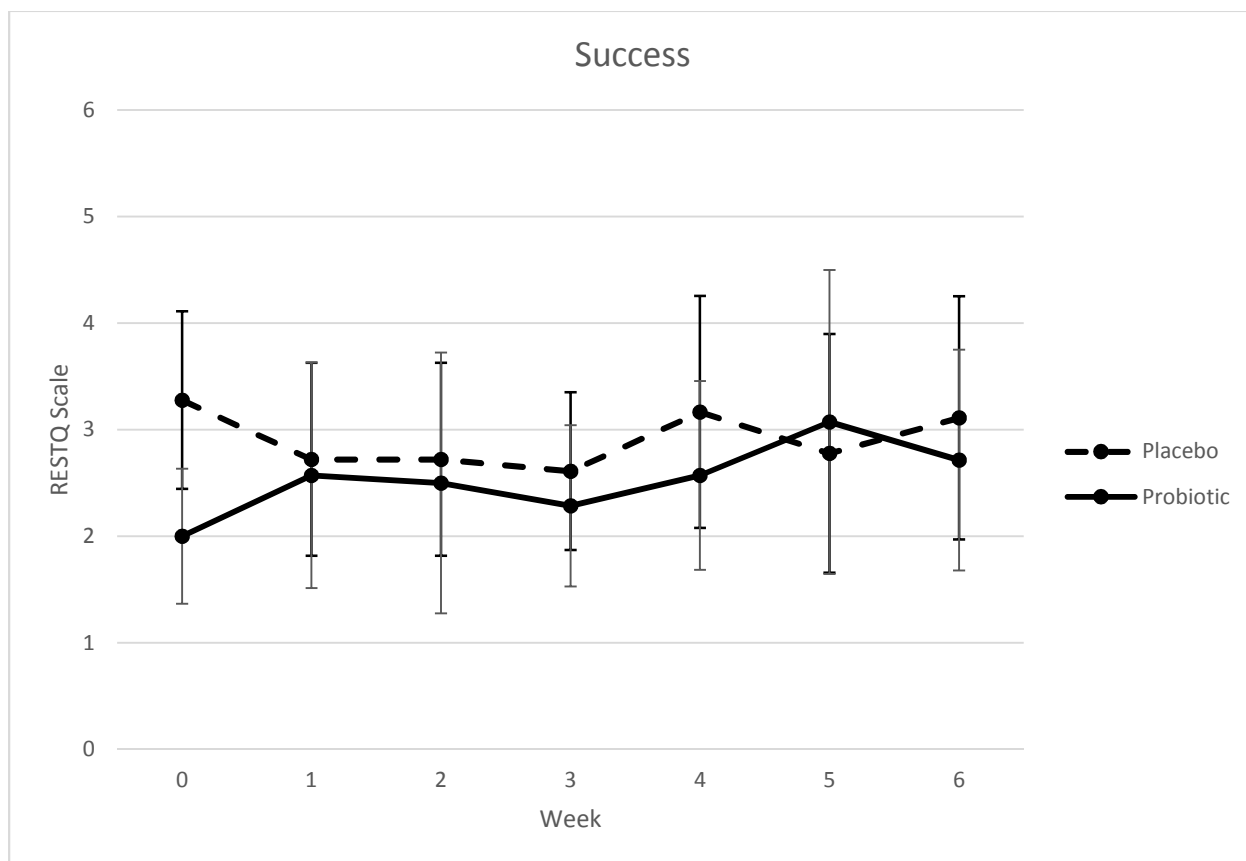
Figure 15 – RESTQ52-Sport Scale – Success

Figure 15 RESTQ52-Sport weekly success scores in collegiate female swimmers in 6 weeks of exercise training and treatment. Success, pleasure at work, and creativity during the past few days are assessed in this area. RESTQ52-Sport scale is defined as 0-never, 1-seldom, 2-sometimes, 3-often, 4-more often, 5-very often, 6-always. *Probiotic* the *B. infantis*35624 supplemented group, *Placebo* group, *Week* 0-6; n = 7 (*B. infantis*35624 supplementation), n = 9 (placebo). Values are means \pm SD.

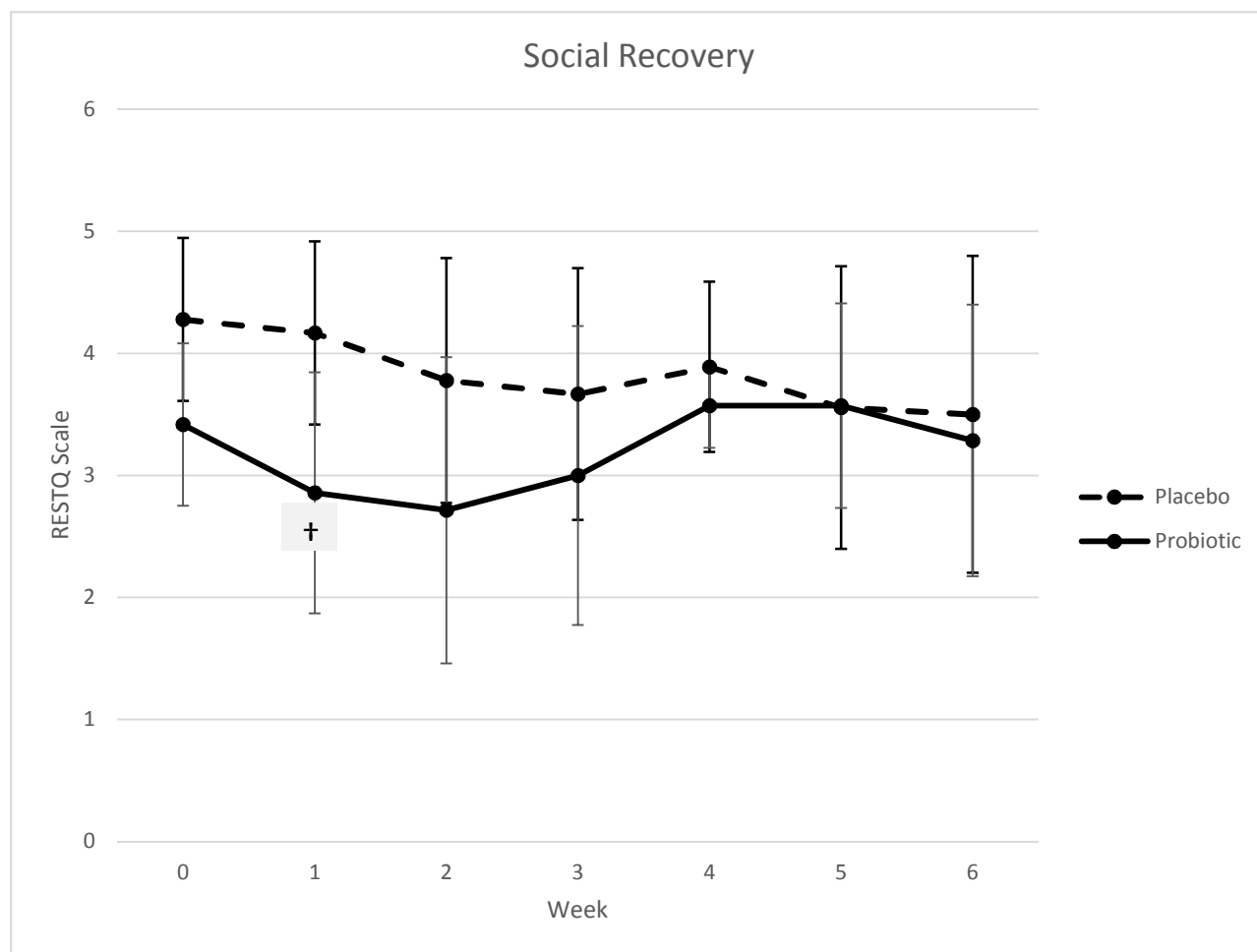
Figure 16 – RESTQ52-Sport Scale – Social Recovery

Figure 16 RESTQ52-Sport weekly social recovery scores in collegiate female swimmers in 6 weeks of exercise training and treatment. High values are shown by athletes who have frequent pleasurable social contacts and change combined with relaxation and amusement. RESTQ52-Sport scale is defined as 0-never, 1-seldom, 2-sometimes, 3-often, 4-more often, 5-very often, 6-always. *Probiotic* the *B. infantis*35624 supplemented group, *Placebo* group, *Week* 0-6; n = 7 (*B. infantis*35624 supplementation), n = 9 (placebo). Values are means ± SD. There was a trend towards significant difference between groups at week 1 of exercise training and treatment: † P < 0.10 (ANCOVA).

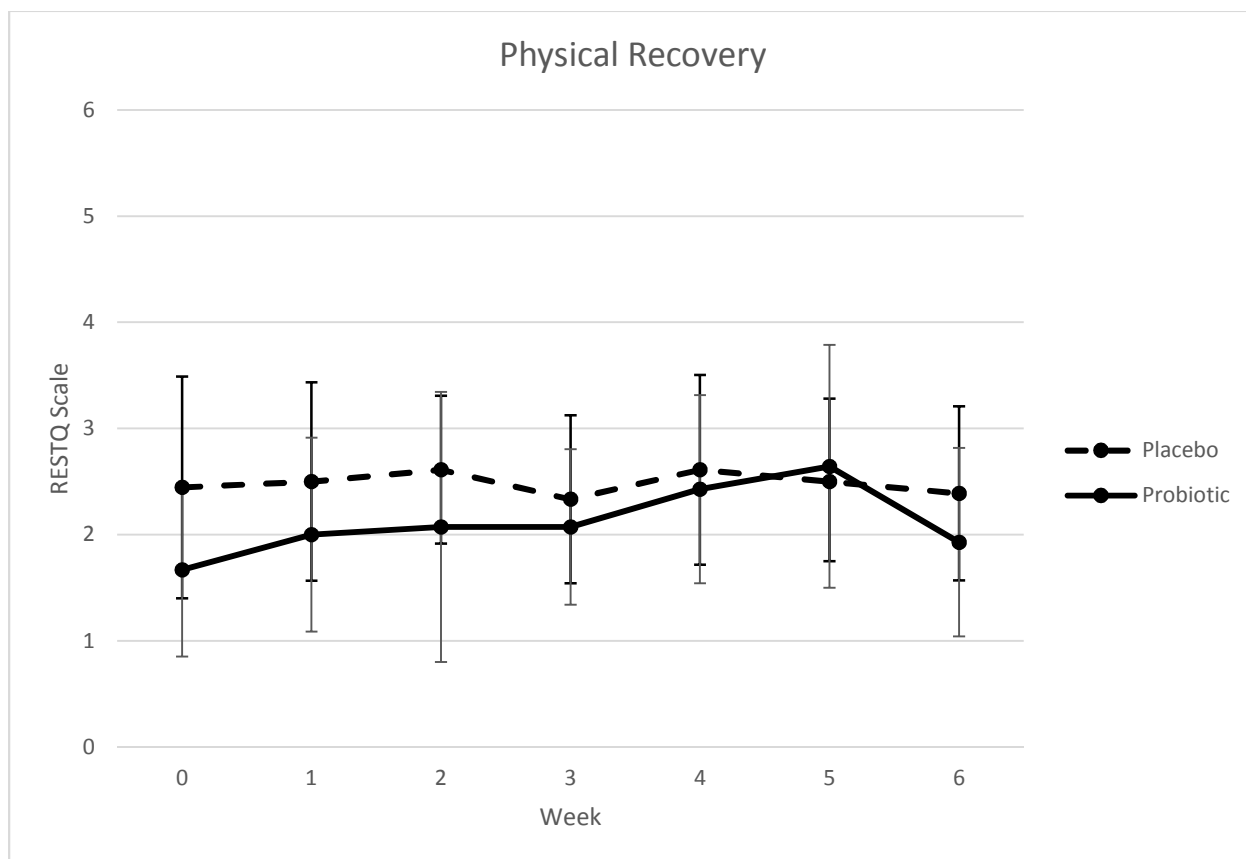
Figure 17 – RESTQ52-Sport Scale – Physical Recovery

Figure 17 RESTQ52-Sport weekly physical recovery scores in collegiate female swimmers in 6 weeks of exercise training and treatment. Physical recovery, physical well-being, and fitness are characterized in this area. RESTQ52-Sport scale is defined as 0-never, 1-seldom, 2-sometimes, 3-often, 4-more often, 5-very often, 6-always. *Probiotic* the *B. infantis*35624 supplemented group, *Placebo* group, *Week* 0-6; n = 7 (*B. infantis*35624 supplementation), n = 9 (placebo). Values are means \pm SD.

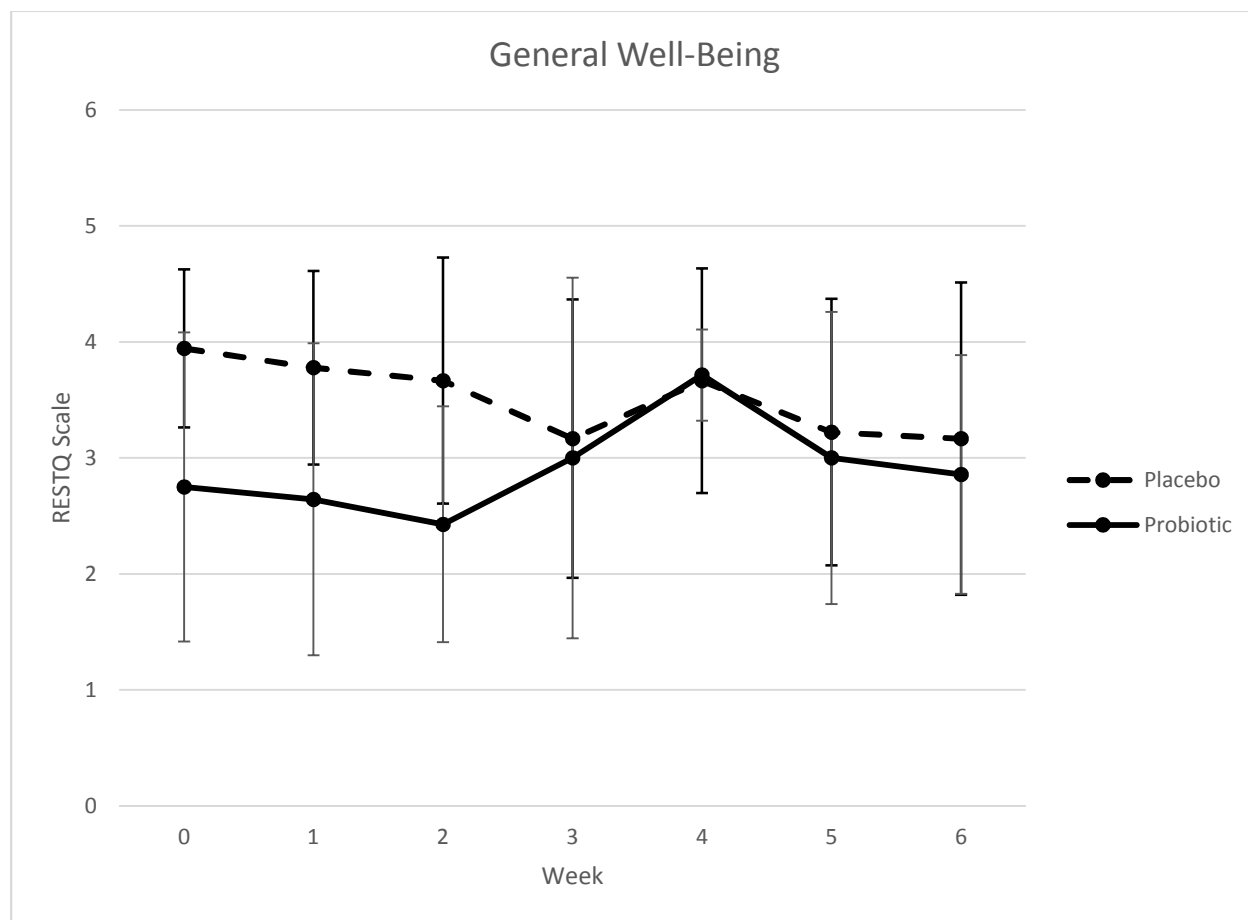
Figure 18 – RESTQ52-Sport Scale – General Well-Being

Figure 18 RESTQ52-Sport weekly general well-being scores in collegiate female swimmers in 6 weeks of exercise training and treatment. Besides frequent good moods and high well-being, general relaxation and contentment are also in this scale. RESTQ52-Sport scale is defined as 0-never, 1-seldom, 2-sometimes, 3-often, 4-more often, 5-very often, 6-always. *Probiotic* the *B. infantis*35624 supplemented group, *Placebo* group, *Week* 0-6; n = 7 (*B. infantis*35624 supplementation), n = 9 (placebo). Values are means \pm SD.

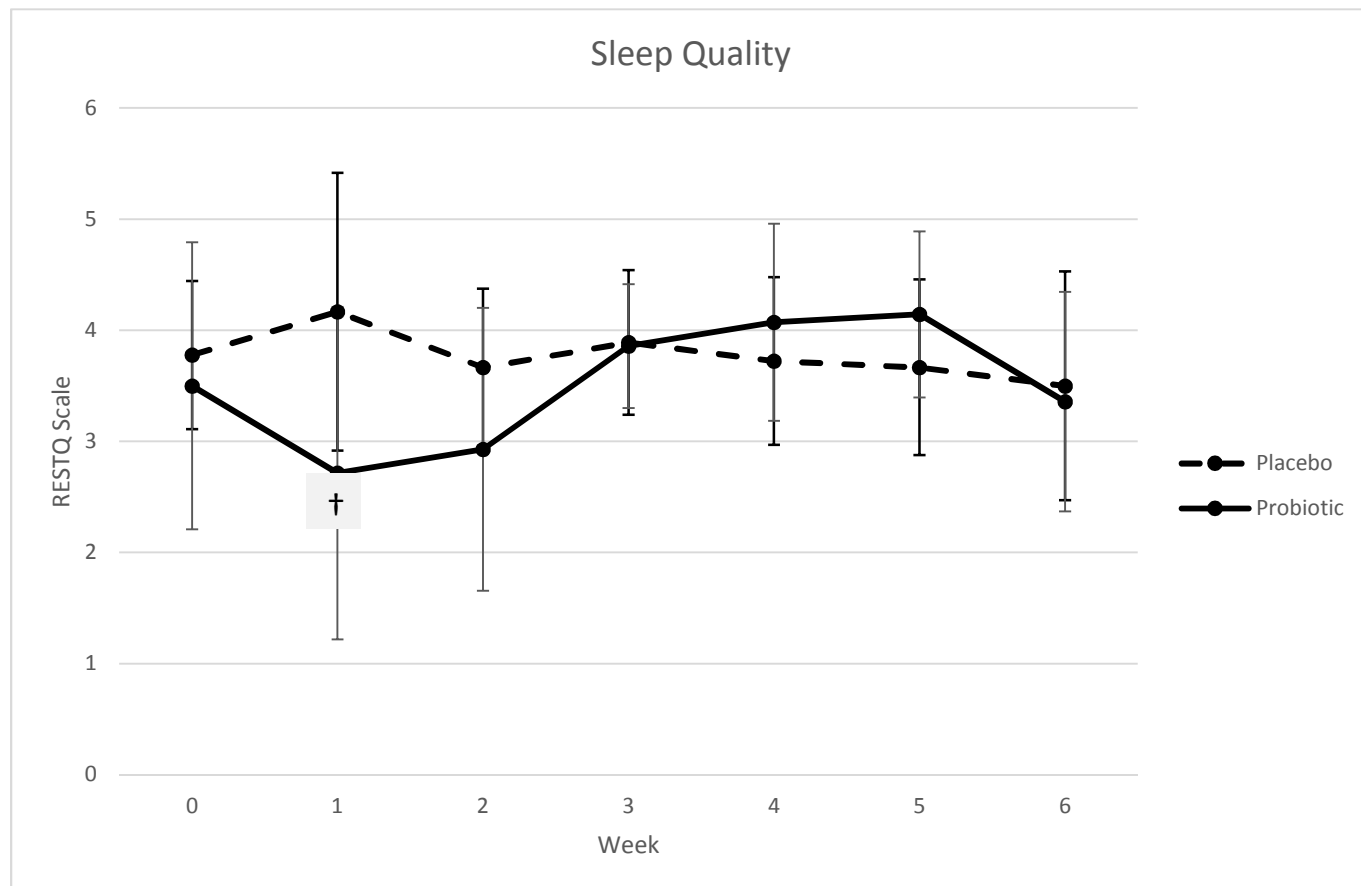
Figure 19 – RESTQ52-Sport Scale – Sleep Quality

Figure 19 RESTQ52-Sport weekly sleep quality scores in collegiate female swimmers in 6 weeks of exercise training and treatment. Enough recovering sleep, an absence of sleeping disorders while falling asleep, and sleeping through the night characterize recovery sleep. RESTQ52-Sport scale is defined as 0-never, 1-seldom, 2-sometimes, 3-often, 4-more often, 5-very often, 6-always. *Probiotic* the *B. infantis*35624 supplemented group, *Placebo* group, *Week* 0-6; n = 7 (*B. infantis*35624 supplementation), n = 9 (placebo). Values are means ± SD. There was a trend towards significant difference between groups at week 1 of exercise training and treatment: † P < 0.10 (ANCOVA).

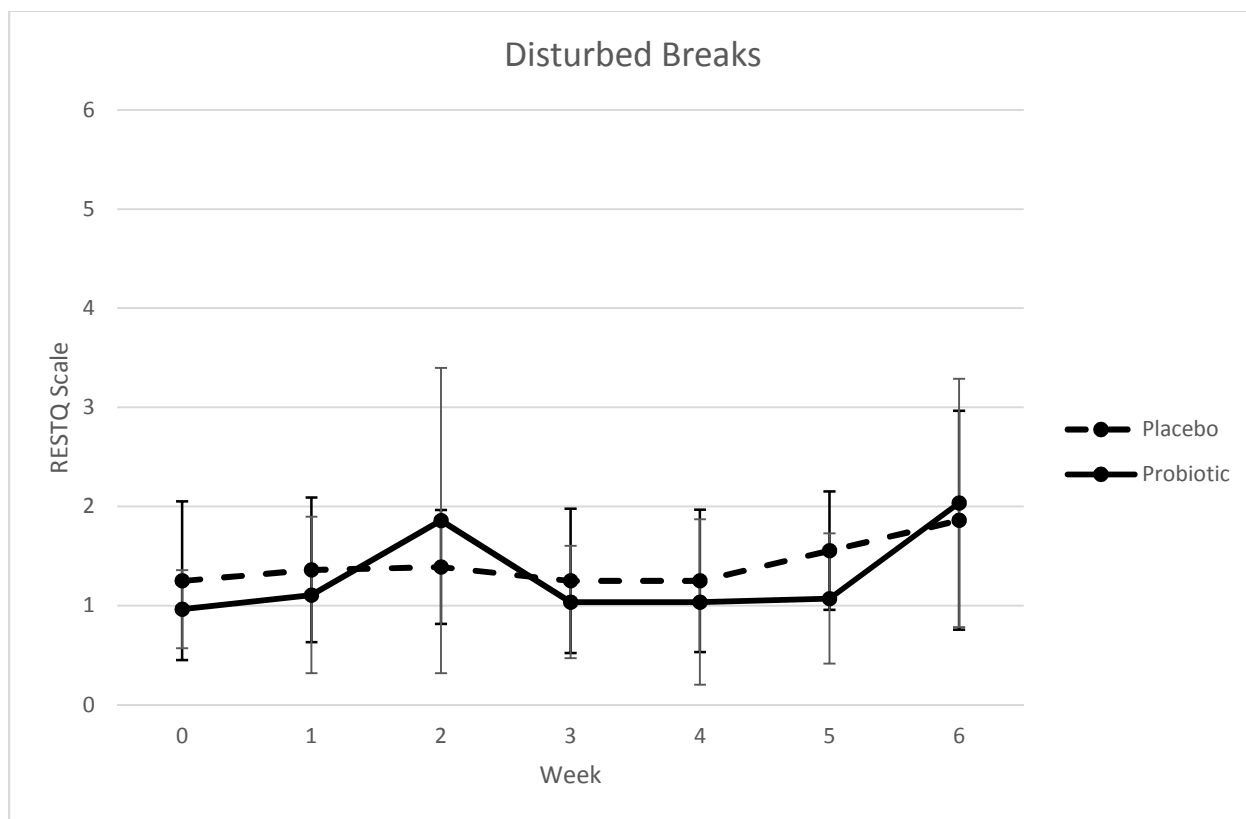
Figure 20 – RESTQ52-Sport Scale – Disturbed Breaks

Figure 20 RESTQ52-Sport weekly sleep quality scores in collegiate female swimmers in 6 weeks of exercise training and treatment. This scale deals with recovery deficits, interrupted recovery, and situational aspects that get in the way during periods of rest (e.g., teammates, coaches). RESTQ52-Sport scale is defined as 0-never, 1-seldom, 2-sometimes, 3-often, 4-more often, 5-very often, 6-always. *Probiotic* the *B. infantis*35624 supplemented group, *Placebo* group, *Week* 0-6; n = 7 (*B. infantis*35624 supplementation), n = 9 (placebo). Values are means \pm SD.

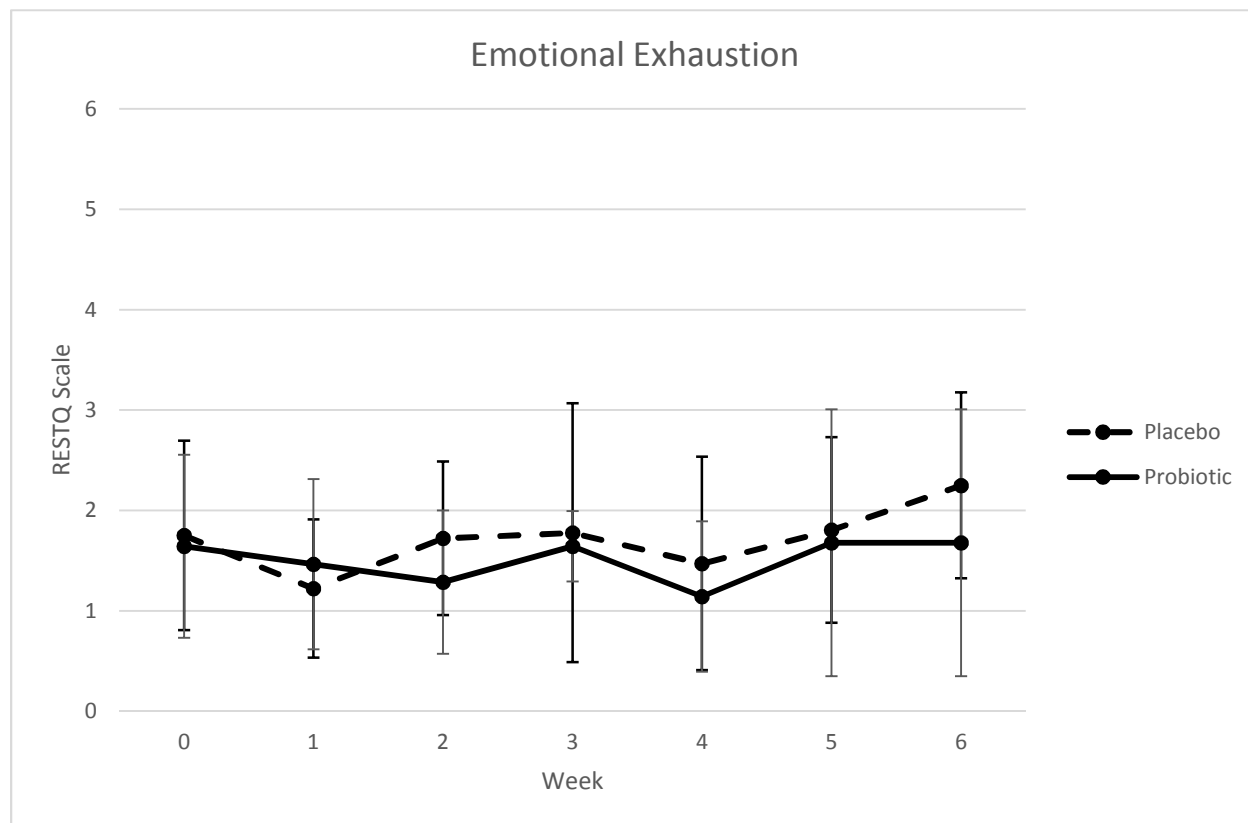
Figure 21 – RESTQ52-Sport Scale – Emotional Exhaustion

Figure 21 RESTQ52-Sport weekly emotional exhaustion scores in collegiate female swimmers in 6 weeks of exercise training and treatment. High scores are shown by athletes who feel burned out and want to quit their sport. RESTQ52-Sport scale is defined as 0-never, 1-seldom, 2-sometimes, 3-often, 4-more often, 5-very often, 6-always. *Probiotic* the B. infantis35624 supplemented group, *Placebo* group, Week 0-6; n = 7 (B. infantis35624 supplementation), n = 9 (placebo). Values are means \pm SD.

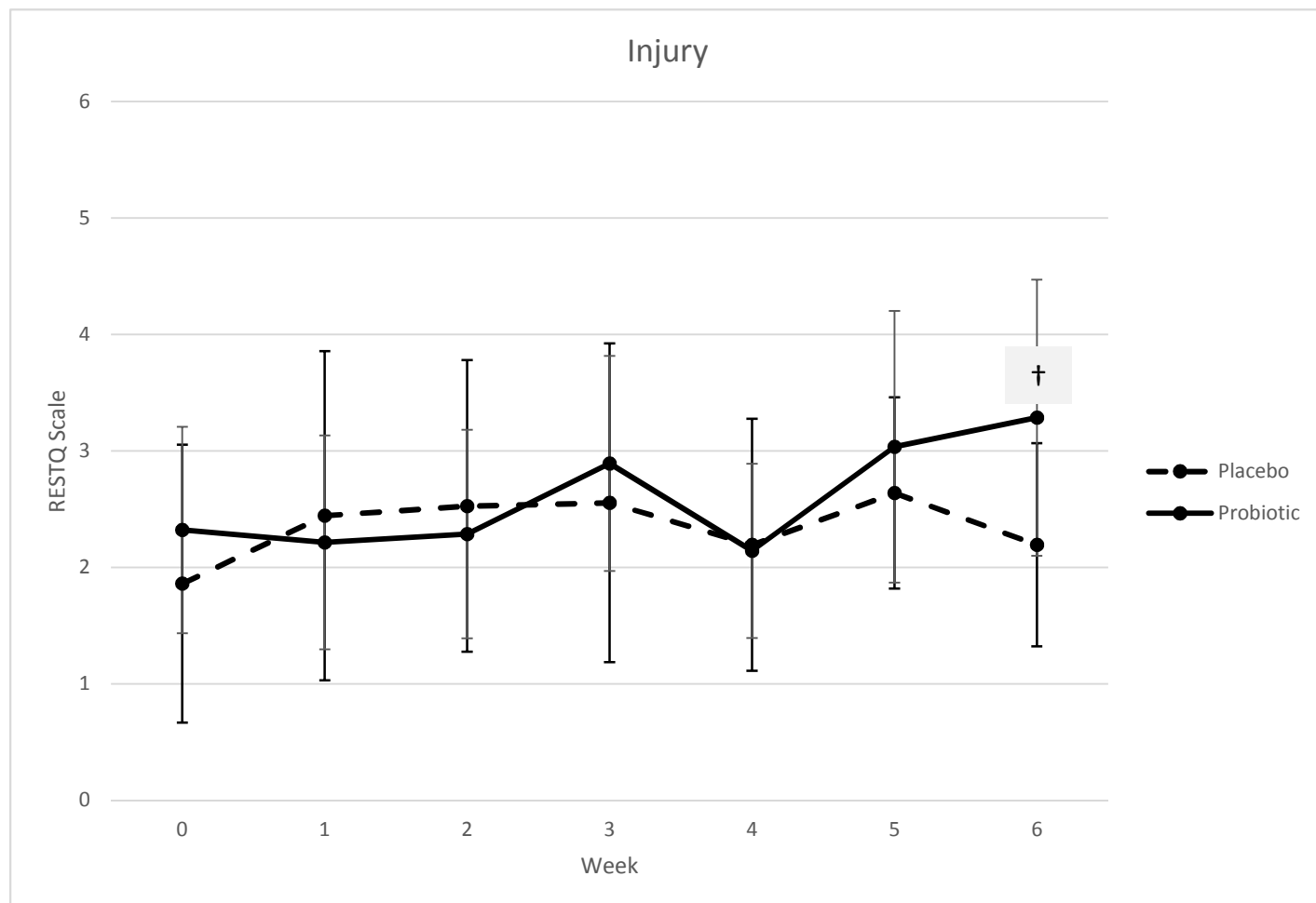
Figure 22 – RESTQ52-Sport Scale – Injury

Figure 22 RESTQ52-Sport weekly injury scores in collegiate female swimmers in 6 weeks of exercise training and treatment. High scores signal an acute injury or vulnerability to injuries. RESTQ52-Sport scale is defined as 0-never, 1-seldom, 2-sometimes, 3-often, 4-more often, 5-very often, 6-always. *Probiotic* the *B. infantis*35624 supplemented group, *Placebo* group, Week 0-6; n = 7 (*B. infantis*35624 supplementation), n = 9 (placebo). Values are means ± SD. There was a trend towards significant difference between groups at week 6 of exercise training and treatment: † P < 0.10 (ANCOVA).

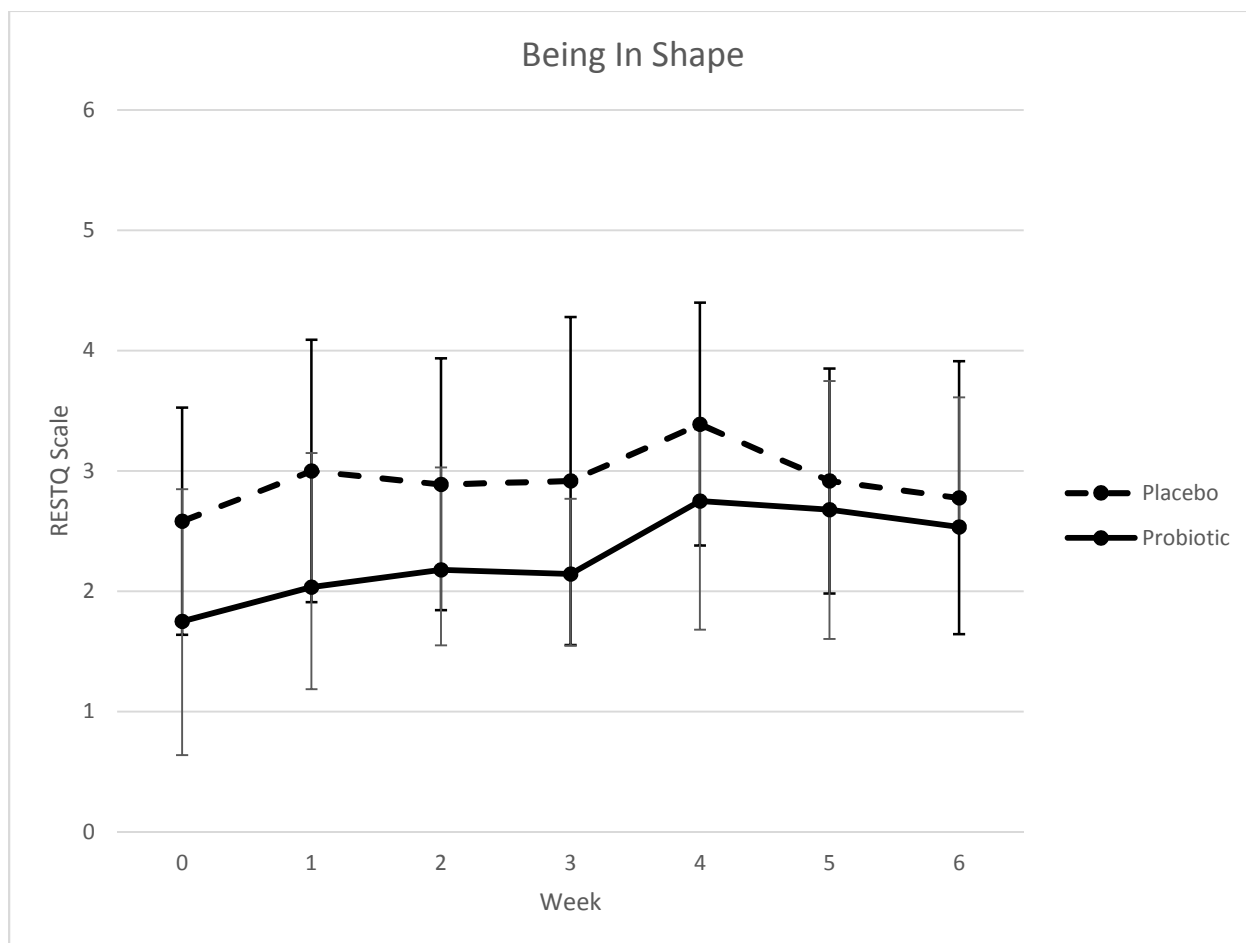
Figure 23 – RESTQ52-Sport Scale – Being in Shape

Figure 23 RESTQ52-Sport weekly being in shape scores in collegiate female swimmers in 6 weeks of exercise training and treatment. Athletes with high scores describe themselves as fit, physically efficient, and vital. RESTQ52-Sport scale is defined as 0-never, 1-seldom, 2-sometimes, 3-often, 4-more often, 5-very often, 6-always. *Probiotic* the B. infantis35624 supplemented group, *Placebo* group, Week 0-6; n = 7 (B. infantis35624 supplementation), n = 9 (placebo). Values are means \pm SD.

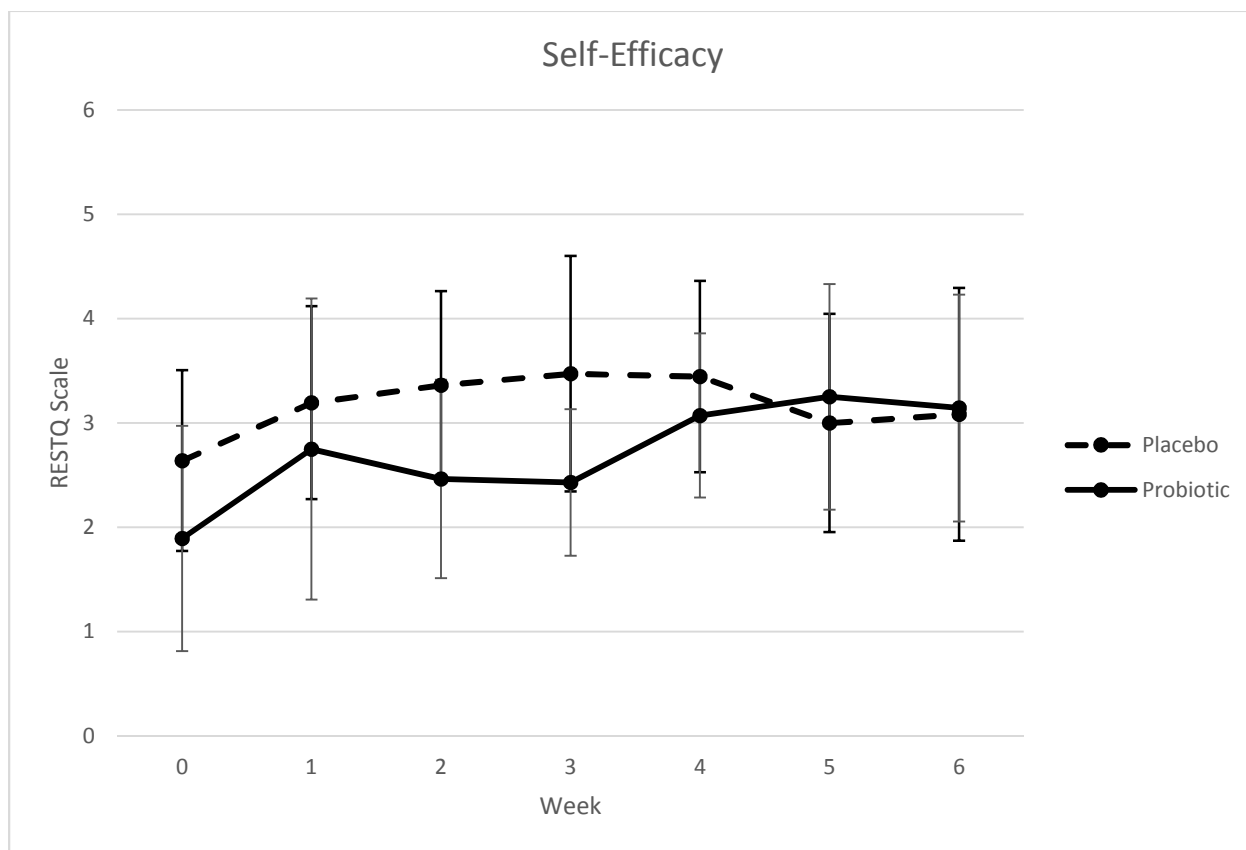
Figure 24 – RESTQ52-Sport Scale – Self-Efficacy

Figure 24 RESTQ52-Sport weekly self-efficacy scores in collegiate female swimmers in 6 weeks of exercise training and treatment. This scale is characterized by how convinced the athlete is that he/she has trained well and is optimally prepared. RESTQ52-Sport scale is defined as 0-never, 1-seldom, 2-sometimes, 3-often, 4-more often, 5-very often, 6-always. *Probiotic* the *B. infantis*35624 supplemented group, *Placebo* group, *Week* 0-6; n = 7 (*B. infantis*35624 supplementation), n = 9 (placebo). Values are means \pm SD.

Table 1 – Dietary Food Log ANOVA Analysis

	Placebo Supplementation			Probiotic Supplementation		
	Week 0 (n=9)	Week 3 (n=9)	Week 6 (n=9)	Week 0 (n=7)	Week 3 (n=8)	Week 6 (n=8)
Total Energy (calories) (min - max)	1776 ± 445 (1076 - 2493)	2069 ± 430 (1493 - 2727)	1939 ± 415 (1401 - 2605)	2284 ± 536 (1742 - 3252)	2454 ± 1064 (1690 - 4996)	2540 ± 806 (1585 - 4122)
Carbohydrates (grams) (min - max)	194 ± 63 (107 - 282)	216 ± 68 (156 - 372)	215 ± 78 (125 - 382)	254 ± 92 (163 - 438)	285 ± 160 (171 - 669)	297 ± 119 (178 - 551)
Protein (grams) (min - max)	105 ± 44 (67 - 213)	119 ± 45 (62 - 182)	126 ± 44 (81 - 216)	130 ± 31 (71 - 161)	138 ± 52 (97 - 259)	131 ± 34 (93 - 187)
Fat (grams) (min - max)	68 ± 17 (34 - 92)	84 ± 26 (54 - 136)	69 ± 21 (46 - 97)	87 ± 29 (55 - 136)	93 ± 37 (62 - 169)	98 ± 35 (51 - 150)

	Significance Between Groups (p-value)		
	Week 0	Week 3	Week 6
	Robust Test of Equality - Welch		
Total Energy	0.066	0.363	0.087
Carbohydrates	0.169	0.283	0.126
Protein	0.201	0.435	0.781
Fat	0.16	0.569	0.07